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THE EFFECT OF THE ADMINISTRATION OF *p*-DIMETHYLAMINOAZOBENZENE ON THE OVARIAN FUNCTIONS OF RATS.

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Biskind and Biskind (1942) demonstrated that the capacity of the liver of normal rats to inactivate oestrogens was impaired by a vitamin B deficient diet. Shipley and György (1944) found that rat liver damaged by low-protein high-fat diet was unable to inactivate oestrone. Unna, Singher, Kensler, Taylor and Rhoads (1944) found a correlation between the loss of riboflavin content of the liver and the loss of the capacity for inactivating oestrone in rats maintained on a low protein diet.

The efficacy of *p*-dimethylaminoazobenzene in producing tumours of the liver is greatly influenced by dietary factors; the lack of vitamin B complex and/or protein seem to be the most important factors (Rhoads, 1940; Kensler, Sugiura, Young, Halter and Rhoads, 1941, reviewed by Burk, 1944).

It might therefore be expected that substances which damage the liver to the extent of tumour formation would also affect its property of destroying endogenous oestrogen, thus provoking prolonged or permanent oestrus. The present experiment reports the effect of the administration of *p*-dimethylaminoazobenzene on the oestral cycle of the rat.

EXPERIMENTAL.

Female albino rats weighing about 100 g. at the beginning of the experiment were used. All animals were kept in the same room. Polished rice and water were given to all rats *ad lib.* Nine groups of animals received additions as follows (Table I).

TABLE I.—*Supplements Added Daily to the Polished Rice Basal Diet.*

Experiment started.	Addition.	Number of animals.
12.vii.44	Carrots + 2% arachis oil	10
12.vii.44	„ + 2% „ + 0·06% *p*-dimethylaminoazobenzene	20
23.xi.44	6% Casein + 2% arachis oil + carrots	10
23.xi.44	6% „ + 2% „ + „ + 0·06% *p*-dimethylaminoazobenzene	30
1.xii.43	†10 c.c. milk + 2% arachis oil*	20
1:xii.43	10 c.c. „ + 2% „ + 0·06% *p*-dimethylaminoazobenzene*	20
3.iv.45	10 c.c. „ + 2% „ + 0·06% „ *	20
20.x.44	Carrots	10
20.x.44	„ + 2% arachis oil + 0·06% azobenzene	10

* Greens once weekly.
† Milk was supplied in bulk and allowed 10 c.c. for each rat.

From 10 animals of each group, selected at random at the onset of the experiment, vaginal smears were taken daily at 2 p.m., except on Saturdays and Sundays, until the animal was killed, and examined microscopically. The animals were weighed weekly. Rats were killed at intervals, and the weights of the organs were taken immediately after killing the animal. Histological sections were obtained from most of the ovaries.

RESULTS.

Fig. 1, 3 and 6 show that all animals not receiving *p*-dimethylaminoazobenzene exhibited regular oestral cycles during the whole experiment period up to 400 days. Animals receiving *p*-dimethylaminoazobenzene generally became anoestral after about the second week of administering the dye (Fig. 2 and 4). Control animals receiving azobenzene showed some irregularity in the oestral cycles, but there was no permanent anoestrus (Fig. 7). The animals receiving a diet containing *p*-dimethylaminoazobenzene, but supplemented with milk, showed normal oestral cycles (Fig. 5). Experiments carried out on the same animals revealed that the administration of 10 ml. of fresh milk daily prevented

FIGS. 1–7.—Explanation of the signs used:
One horizontal unit represents one day.
Preoestrus is indicated by column of one vertical unit.
Oestrus „ „ three „ units.
Metoestrus „ „ two „ „
Dioestrus „ basic line.

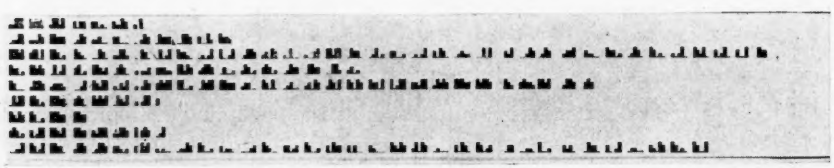


FIG. 1.—Oestral cycles of rats on a diet consisting of rice, carrots and arachis oil.

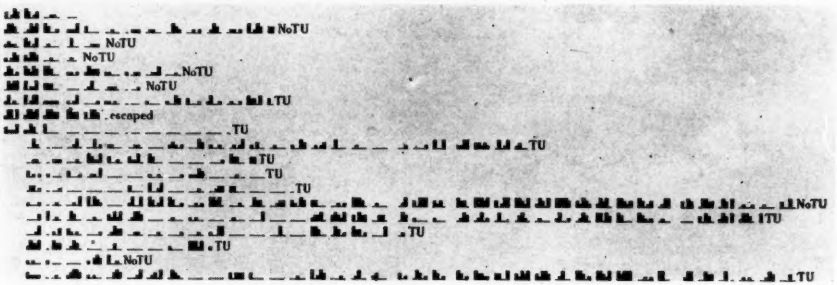


FIG. 2.—Oestral cycles of rats on a diet consisting of rice, carrots, arachis oil and 0·06 per cent *p*-dimethylaminoazobenzene.

Tu. = Tumours in the liver.

FIG. 3.—Oestral cycles of rats on a diet consisting of rice, carrots, casein and arachis oil.

FIG. 4.—Oestral cycles of rats on a diet consisting of rice, carrots, casein, arachis oil and 0·06 per cent *p*-dimethylaminoazobenzene.

Tu. = Tumour in the liver.
↑ = Injections of urinary gonadotrophin started.

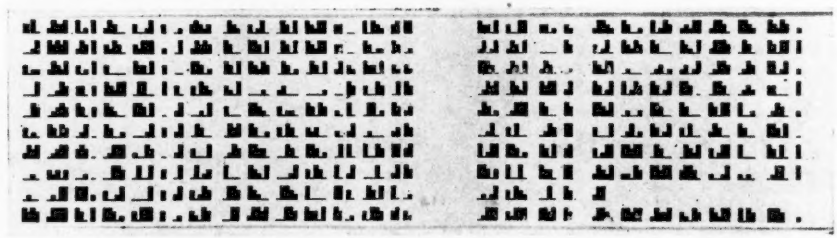


FIG. 5.—Oestral cycles of rats on a diet consisting of rice, arachis oil, milk and 0·06 per cent *p*-dimethylaminoazobenzene.

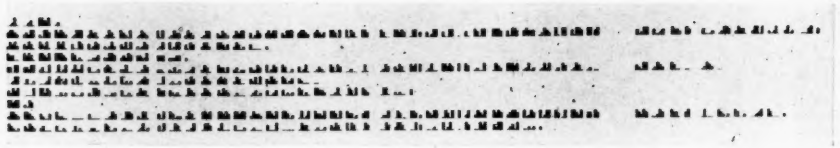


FIG. 6.—Oestral cycles of rats on a diet consisting of rice and carrots.

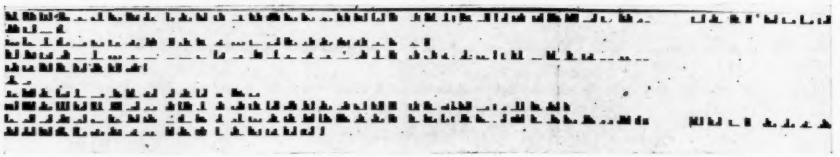


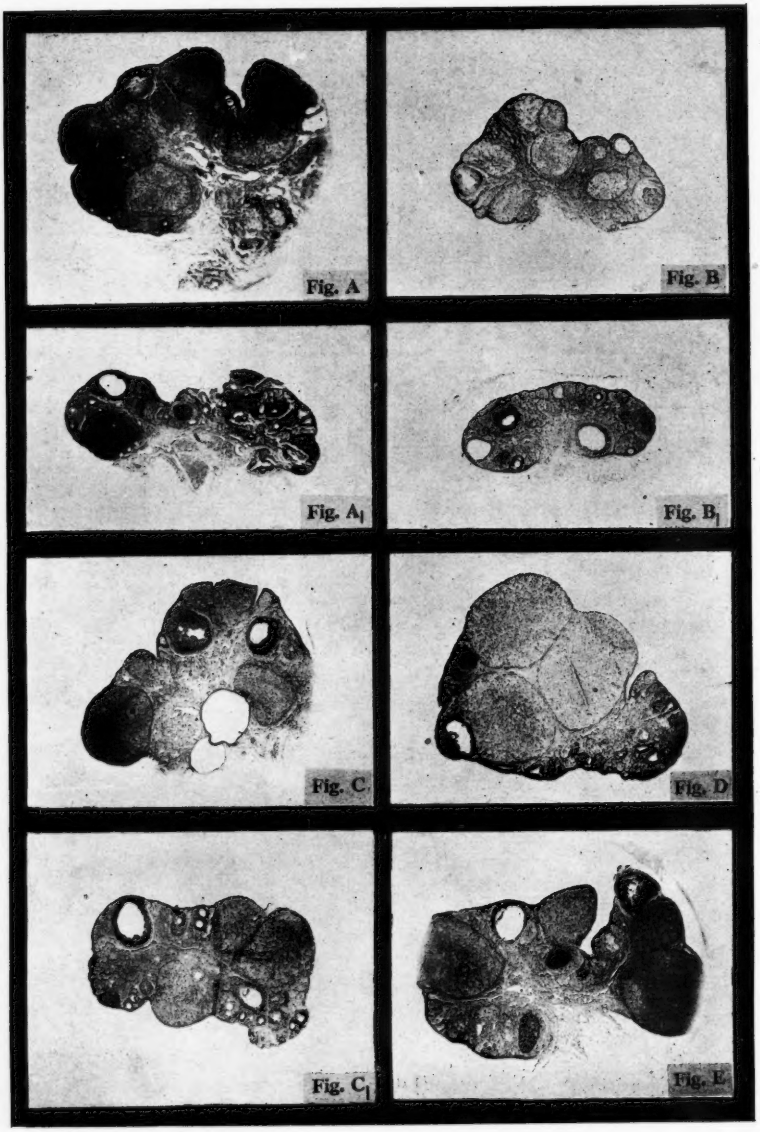
FIG. 7.—Oestral cycles of rats on a diet consisting of rice, carrots, arachis oil and 0·06 per cent azobenzene.

the development of tumours in the liver in 37 out of 40 rats (Hoch-Ligeti, 1946). The absence of the oestral cycles in animals on the tumour-producing diet did not persist indefinitely; in some animals a few oestral cycles occurred during the anoestral period, in others normal oestral cycles reappeared after 5 to 6 months of continuous anoestrus.

TABLE II.—*Ovarian Weights of the Rats.*

Supplement to diet of polished rice.								
Carrots, arachis oil.	Carrots, arachis oil, *p*-dimethyl-aminoazo-benzene.	Carrots, casein, arachis oil.	Carrots, casein, arachis oil, *p*-dimethyl-aminoazo-benzene.	Milk, arachis oil.	Milk, arachis oil, *p*-dimethyl-aminoazo-benzene.	Carrots.	Carrots, arachis oil, azo-benzene.
49·6	38·7	66·3	33·3	100·6*	107·9*	69·3	91·7
44·8	18·6	57·9	53·4	173·4*	105·6*	90·5	67·9
91·7	47·6	46·4	49·8	136·0*	145·0*	67·8	72·1
30·7	22·2	43·6	66·6	115·0*	63·5*	42·2	82·6
45·3	62·7	52·9	16·5	..	..	48·0	95·5
77·6	19·6	90·7	35·9	..	81·6	50·1	64·9
57·4	21·7	60·1	39·2	..	61·4	117·7	70·2
75·0	19·3	68·1	17·8	..	49·6	58·8	55·2
..	33·0	69·7	38·2	..	59·2	86·8	..
..	22·3	..	32·3	..	141·5	..	..
..	59·9	..	31·0	..	107·1	..	..
..	48·5	..	48·0	..	68·2	..	..
..	71·5	..	30·8	..	67·8	..	..
..	..	..	33·9	..	172·1	..	..
..	..	..	40·3	..	110·6	..	..
..	..	..	33·2	..	80·5	..	..
..	..	..	51·3	..	107·0	..	..
..	..	..	40·9	..	74·8	..	..
..	..	..	42·8	..	..	..	..
Number of animals 8	13	9	19	4	17	9	8
Mean 58·9	37·4	61·7	38·7	131·3	94·3	70·1	75·0
Standard error 7·3	5·2	4·8	2·75	15·8	8·4	8·1	4·9

* These animals were over 300 days old when killed.



Hoch-Ligeti.

The ovaries of the animals receiving *p*-dimethylaminoazobenzene, except the animals receiving *p*-dimethylaminoazobenzene plus milk, were smaller than those of the controls (Table II), and histological investigation revealed a nearly complete absence of corpora lutea, arrest of development of primordial follicles and degeneration of the Graafian follicles. The ovaries of rats receiving *p*-dimethylaminoazobenzene plus milk or control diets containing no *p*-dimethylaminoazobenzene were normal. The control animals which were receiving azobenzene also had normal ovaries (Fig. 12).

Drill and Burill (1944) found that a diet deficient in thiamin caused a rapid decrease in the food intake of the rats and cessation of the oestral cycles within 16 to 24 days. Control animals on a diet containing thiamin, given in amounts reduced to such quantities as were consumed by the thiamin-deficient rats, showed the same ovarian response. The ovarian deficiency was apparently caused by the reduced food intake.

Fig. 8 to 11 show that the food intake of animals receiving only the basic rice diet was remarkably lower than that of animals receiving the same diet supplemented with milk. The rate of growth of the animals on the milk diet was about 1·0 g. per day ; growth stopped completely in the absence of milk. The animals on milk plus *p*-dimethylaminoazobenzene consumed less than the controls, but the growth curves do not show any significant difference. The animals receiving *p*-dimethylaminoazobenzene in a diet not containing milk did not consume appreciably less food than their controls (no milk, no *p*-dimethylaminoazobenzene), and they maintained their body weights or showed a slight decrease in weight which was only conspicuous in individual animals at about the time when the hepatic tumour appeared. The reduction in the food intake alone therefore cannot have been responsible for the cessation of the oestral cycles in the present experiment.

Cessation of the oestral cycles occurs (*a*) if the vagina cannot respond to hormonal stimulation, (*b*) if no oestrogenic hormones are produced by the ovaries, or (*c*) if there is lack of production of pituitary gonadotrophic hormones. An attempt was made to determine the cause of the cessation in the present experiments. The ovaries and the pituitary of rats fed on *p*-dimethylaminoazobenzene on the one hand and of rats not receiving *p*-dimethylaminoazobenzene on the other, were implanted into infantile mice, and the ovarian response studied. The transplantation of only one pituitary from a rat into infantile mice did not have any definite effect on the ovaries of the host. The transplantation of the ovaries from a control rat (rice-carrot diet) into an infantile mouse (2 cases)

DESCRIPTION OF PLATE.

FIG. 12.—Ovaries of rats on diets with and without addition of *p*-dimethylaminoazobenzene.

Diet to A : rice, carrots, arachis oil for 56 days.
„ A_1 : „ „ „ + *p*-dimethylaminoazobenzene for 47 days.

Diet to B : rice, carrots, arachis oil, casein for 189 days.
„ B_1 : „ „ „ „ + *p*-dimethylaminoazobenzene for 48 days.

Diet to C : rice, arachis oil, milk for 471 days.
„ C_1 : „ „ „ + *p*-dimethylaminoazobenzene for 78 days.

Diet to D : rice, carrots, for 78 days.
„ E : „ „ arachis oil + azobenzene for 57 days.

Magnification : × 12.

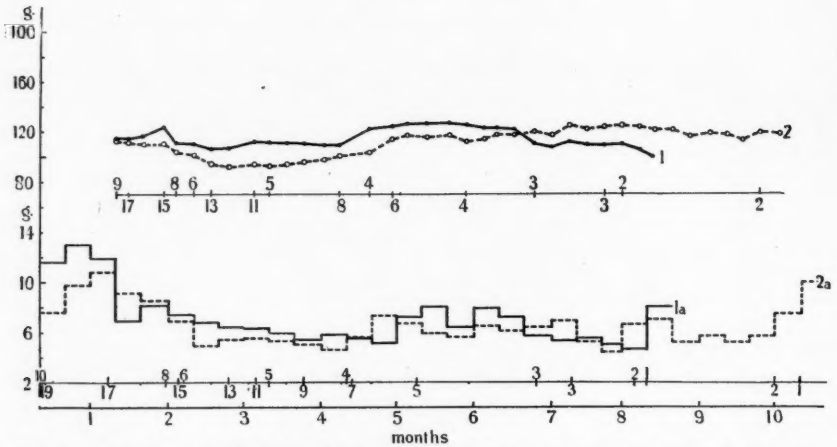


FIG. 8.—Growth and food intake of rats on diet consisting of rice, carrots and 2 per cent arachis oil with and without addition of *p*-dimethylaminoazobenzene.

○ ——— ○ Animals on basic diet.
○ - - - - ○ Animals receiving addition of *p*-dimethylaminoazobenzene.

The upper two curves (1 and 2) represent the means of body weights. The lower two curves (1*a* and 2*a*) represent the average food intake during ten-day periods per animal per day. The figures along the lines below each pair of curves represent the number of rats surviving in the corresponding groups. The figures above the line refer to the animals on basic diet, and the numbers below the line to the animals receiving *p*-dimethylaminoazobenzene.

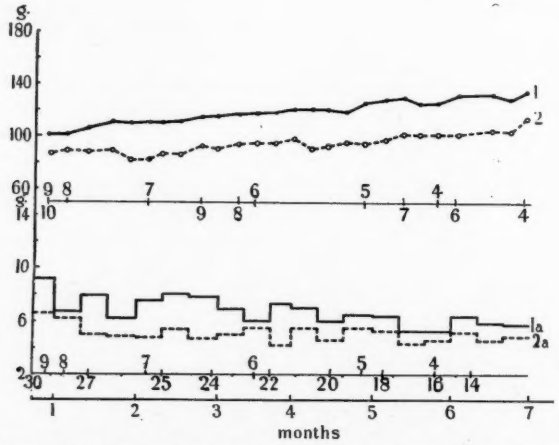


FIG. 9.—Growth and food intake of rats on diet consisting of rice, 6 per cent casein, carrots and 2 per cent arachis oil with and without addition of *p*-dimethylaminoazobenzene.

○ ——— ○ Animals on basic diet.
○ - - - - ○ Animals receiving addition of *p*-dimethylaminoazobenzene.

The upper two curves (1 and 2) represent the means of body weights. The lower two curves (1*a* and 2*a*) represent the average food intake during ten-day periods per animal per day. The figures along the lines below each pair of curves represent the number of rats surviving in the corresponding groups. The numbers above the line refer to the animals on basic diet and the numbers below the line to the animals receiving *p*-dimethylaminoazobenzene.

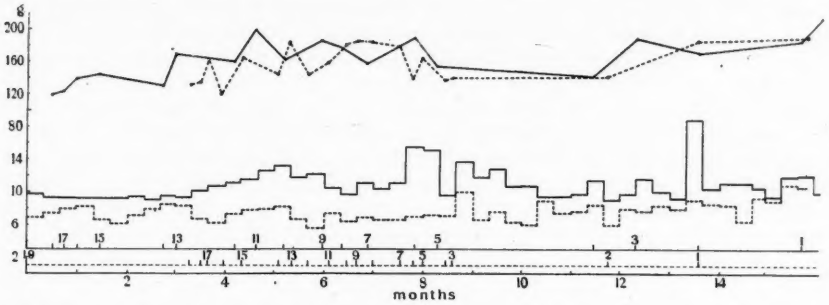


FIG. 10.—Growth and food intake of rats on diet consisting of rice, 2 per cent arachis oil and milk with and without addition of *p*-dimethylaminoazobenzene.

○ ——— ○ Animals on basic diet.
○ - - - - ○ Animals receiving addition of *p*-dimethylaminoazobenzene.

The upper two curves (1 and 2) represent the body weight of individual animals on the day when they were killed. The lower two curves (1*a* and 2*a*) represent the average food intake during ten-day periods per animal per day. The numbers along the straight lines (1*b* and 2*b*) represent the number of rats surviving in the corresponding groups.

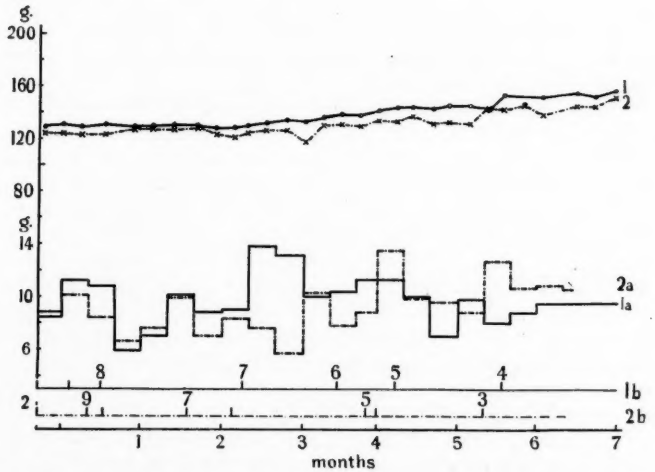


FIG. 11.—Growth and food intake of rats on diet consisting of rice and carrots with and without addition of azobenzene in arachis oil.

○ ——— ○ Animals on basic diet.
× - - - - × Animals receiving addition of azobenzene in arachis oil.

The upper two curves (1 and 2) represent the means of body weights. The lower two curves (1*a* and 2*a*) represent the average food intake during ten-day periods per animal per day. The numbers along the straight lines (1*b* and 2*b*) represent the number of rats surviving in the corresponding groups.

resulted in the opening of the vagina, in an increased uterine weight after 100 hours, and in ripening the follicles as seen microscopically. The ovaries of the animals treated with *p*-dimethylaminoazobenzene seemed to contain no oestrogenic hormone (2 cases).

Since a limited number of transplantations of simple pituitaries did not seem to yield clear-cut results as to the functional state of the pituitary, the effect of hormone injections was investigated. Urinary gonadotrophin (15 rat units per day) was injected into rats receiving *p*-dimethylaminoazobenzene after they had been anoestral for 6 to 7 months (Fig. 4). A prompt oestral reaction occurred, and the size of the ovaries increased to 150–250 mg. Histologically the presence of 12 to 15 corpora lutea and many ripening follicles could be seen. The vaginal response disappeared after about 3 to 4 weeks in spite of daily administrations of the gonadotrophic hormone. The size of the ovaries decreased also.

DISCUSSION.

The finding in the present experiments that the oestral cycles disappeared in animals in which the liver was damaged by administration of *p*-dimethylaminoazobenzene is apparently in contradiction to the observation of previous workers, where liver damage due to poisons or inadequate dietary regime resulted in a decreased inactivation of oestrogen. The discrepancy was cleared up by demonstrating that, in the animals in the experiment described here on a diet consisting of rice or rice and 6 per cent casein, on addition of 0·06 per cent *p*-dimethylaminoazobenzene no oestrogenic hormone is formed owing to disturbance of the production of gonadotrophic hormone. On administration of urinary gonadotrophin the ovaries of these rats previously rendered anoestral for several months responded in 48 hours in the same way as ovaries of infantile rodents would do, i.e. by follicle ripening and formation of corpora lutea. It has been established (Collip, Selye and Thomson, 1933; Evans, Meyer and Simpson, 1932; Fluhman, 1934) that the urinary gonadotrophin acts through the pituitary of the animals. The prompt reaction of the rats to urinary gonadotrophin together with the spontaneous recurrence of oestral cycles indicates that the activity of the pituitary is suppressed, but that the gland itself is not irreversibly damaged.

It is remarkable that while both the animals treated with *p*-dimethylaminoazobenzene and the control animals on the same diet without *p*-dimethylaminoazobenzene cease to grow, only the rats treated with *p*-dimethylaminoazobenzene lose their oestral cycles. The interruption of the growth might be due to the reduced food intake on an inadequate diet. The reduction of the caloric intake alone cannot be the determining factor, since the animals on the diet containing *p*-dimethylaminoazobenzene, but supplemented with milk, do not consume more food than the animals on basic food without milk and without *p*-dimethylaminoazobenzene, and yet grow normally. It seems that the lack of vitamins and/or a consequent hormonal disturbance might be responsible. Rats on the rice diet supplemented with fresh milk, with or without *p*-dimethylaminoazobenzene, grow at the same rate, although the food intake of the animals receiving *p*-dimethylaminoazobenzene is about 30 per cent less. A reduced activity of the animals receiving *p*-dimethylaminoazobenzene might explain this anomaly. On the other hand hormonal influences might be also involved.

A reduction of the thyrotrophic hormone would result in a decrease of the basic metabolic rate, and consequently in lower food intake with normal growth rate.

Although none of the animals which developed liver tumours when *p*-dimethylaminoazobenzene was administered had regular oestral cycles during the experiment, no strict relation could be established between the onset and duration of the anoestral period and the speed of the development of the tumours. Some of the animals of the group receiving rice, carrots and *p*-dimethylaminoazobenzene regained their oestral cycles after 3 to 5 months and developed tumours, and one anoestral rat of the rice-casein-*p*-dimethylaminoazobenzene group killed on the 330th day of the experiment had no tumour in the liver.

SUMMARY.

1. The oestral cycles of rats cease within 14 to 21 days when 0·06 per cent. *p*-dimethylaminoazobenzene is added to diets consisting of rice and carrots or rice, casein (6 per cent) and carrots. 90 to 95 per cent of the animals on these diets surviving 90 days and over developed tumours in the liver.

2. When the diet containing *p*-dimethylaminoazobenzene is supplemented with fresh milk the oestral cycles remain normal, and the development of tumours is retarded and reduced to 7·5 per cent.

3. Animals on the basal diets without *p*-dimethylaminoazobenzene have normal oestral cycles.

4. Azobenzene caused a transitory disturbance of the oestral cycles beginning 14 to 21 days after administration started.

5. The cessation of oestral cycles is caused by a disturbance of the production of gonadotrophic hormone of the pituitary.

I am indebted to Mr. Harold Burrows for his help in the examination of the histological sections of the ovaries. I wish to express my gratitude to the Sir Halley Stewart Trust for a fellowship held during this work. Also, my thanks are due to the British Empire Cancer Campaign, the Anna Fuller Fund, and to the Jane Coffin Childs Memorial Fund for grants made towards the support of the work carried out in The Chester Beatty Research Institute. I also wish to thank Miss M. I. Houchin for her technical assistance.

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STUDIES ON THE DETOXICATION OF THE LABILE RICKETTSIA TOXIN.

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KLIGLER and Oleinik (1944) have described the existence of a labile toxin in yolk-sac cultures of rickettsiae. Intraperitoneal injection of 0·2 ml. of this toxin into mice or rats regularly induced after three to five days a considerable enlargement of both the spleen and liver. This toxin was found present in cultures of both *R. prowazeki* and *R. mooseri*. The experiments reported below were undertaken in the course of a search for methods of effecting detoxication and of producing immunity to the effects of this toxin.

METHODS.

The labile toxin was prepared by centrifugation from the supernatant fluid of yolk-sac cultures as described by Kligler and Oleinik (1944), as well as by filtration through Seitz and Mandler filters. This toxin was then injected intra-abdominally into white rats weighing 15 g. and 14 days old. It was necessary to employ very young animals, because older rats become relatively insensitive to the toxin, and so fail to show splenic enlargement with sufficient regularity. For each material at least four rats were used. Four days after injection the animals were sacrificed and the average spleen weight was determined for each group.

RESULTS.

Filterability of the Toxin.

Since the toxic effects described by Kligler and Oleinik were produced with suspensions of living rickettsiae or with the supernatant fluids obtained after centrifugation of yolk-sac cultures, filtration experiments were undertaken in order to ensure that the toxic substances under question are real filtrable toxins, and that the toxic effects observed are not associated with the presence of living micro-organisms. The toxic effects of the filtrates injected into white rats compared with those of the supernatant fluid after centrifugation are summarized in Table I.

Stability of Toxin.

(a) *Effect of pH.*—Further experiments were undertaken in order to determine for how long a time the toxin may be kept in different substrates in an ice box without loss of toxicity.

TABLE I.—*Spleen Enlargement of White Rats Weighing* 15 *g. Four Days after Injection of Rickettsial Toxins Prepared by Different Methods. Quantity Injected* 0·2 *c.c.*

Toxin prepared by—	Mode of injection.	Average weight of spleen. (mg.)
Filtration through—		
Mandler filter (test 6 lb.)	i.p.	200
	s.c.	140
,, ,, (test 13 lb.)	i.p.	230
	s.c.	170
Seitz-filter	i.p.	170
	s.c.	140
Centrifugation (supernatant fluid)	i.p.	260
	s.c.	200
	Untreated	70

Stability of toxin was determined in different glycocol-NaOH or glycocol-HCl buffer solutions prepared according to Soerensen. One part toxin was diluted with nine parts buffer and stored in the ice box for different periods. The results of these experiments are summarized in Table II.

TABLE II.—*Effect of pH on the Rickettsial Toxin. Toxin Diluted* 1 : 10 *in Different Buffer Solutions.*

Time of exposure (days).	Average weight of spleen (mg.) in rats.							
	Untreated.	Inoculated with toxin exposed to pH.						
		12·0.	10·0.	7·8.	7·0.	6·1.	4·5.	3·0.
1	60	90	90	120	125	100	105	80
3	50	80	80	100	125	100	90	50
5	55	75	75	100	105	95	55	50
8	50	50	60	60	85	60	50	50
10	60	...	...	70	70	60	...	...
Control : Toxin immediately after the addition of the buffer	60	110	130	140	145	125	130	125

The experiments reported above show that after the addition of the buffer solutions the toxin gradually loses its activity. Around the neutral point (pH 7·8–6·1) this decrease leads to complete inactivity after 8–10 days as described by Kligler and Oleinik (1944). At extremely acid reactions (pH 3·0–4·5) it leads to inactivity after 3–5 days, and at extremely alkaline reactions (pH 10–12) after 5–8 days.

(b) *Effect of various chemical substrates.*—In a further series of experiments various chemical substances were examined for their ability to effect a complete detoxication in as short a time as possible. Rapid detoxication was obtained with formalin, potassium permanganate and sodium bisulphite. To nine parts of the detoxicating solution one part of toxin was added and the mixture kept in the ice box for 24 hours. 0·2 c.c. quantities of the stored mixtures were adminis-

tered intraperitoneally. Table III shows the result of a typical detoxication experiment.

TABLE III.—*Detoxication of Rickettsial Toxin by 24 Hours' Contact in the Ice Box with Formalin, Permanganate or Bisulphite.*

Detoxicating substance employed.	Concentration in per cent.	Deaths after injection.	Average weight of spleen (mg.).
Formalin	0·1	0	50
	0·2	2	50
Permanganate	0·01	0	75
	0·02	0	50
Bisulphite	1·0	0	77
Toxin undiluted	..	0	170
,, diluted 1 : 10	..	0	135
Normal yolk-sac filtrate undiluted	..	0	60
Untreated rats	..	..	60

All the animals which received formalin, permanganate or bisulphite treated toxin showed normal spleen weights, while rats which received untreated toxin showed spleen weights at least twice the normal. The same experiment shows that 0·2 per cent of formalin given intraperitoneally exerts a toxic action. In the following experiments smaller quantities of formalin were therefore employed.

Immunization with Detoxicated Toxins.

Detoxication was effected by substances used in the previous experiment. The rats received two injections at an interval of three days. 0·2 c.c. were given from a mixture which contained one part of toxin and nine parts of the detoxicating solution. Nine days after the last injection all rats received 0·2 c.c. of the undiluted toxin. The rats, which had weighed 15 g. at the beginning of the experiment, had meanwhile grown and weighed 25 g. at the end of the experiment. The following control groups were therefore added: One control group received no type of treatment whatever from the beginning to the end of the experiment. A second group was not immunized, and received an injection of undiluted toxin at the end of the experiment. A third group was immunized with 1 : 10 diluted toxin and was given the undiluted toxin at the end of the experiment.

The experiment represented in Table IV shows that the spleen weights in the different groups of immunized rats are normal or somewhat higher than in the group of the untreated animals. In no group is spleen weight of the animal as high as it is in the group which received toxin without a previous immunization. As a check of the completeness of the detoxication of the immunizing material the effect of different substances was examined on two groups of animals, one with an average weight of 15 g. and the other with an average weight of 25 g. The respective spleen weights 4 days after the second injection of 0·2 c.c. were for the 25 g. animals: untreated, 125 mg.; injected with undiluted toxin, 280 mg.; toxin diluted 1 : 10, 190 mg.; detoxicated material (formalin, permanganate or bisulphite), 125–150 mg. The respective numbers for the rats weighing 15 g. were 90 mg., 270 mg., 180 mg., and 70–150 mg.

TABLE IV.—*Effect of Immunization with Detoxicated Material Given i.p.*

Immunized by toxin treated with—	Concentration of detoxicating substance per cent.	Deaths during immunization.	Average weight of spleen (mg.).
Formalin	0·05	0	184
	0·1	1	150
	0·2	3	100
Permanganate	0·01	0	162
	0·02	0	137
	0·04	0	100
Bisulphite	1·0	0	137
	2·0	0	137
Control immunized with toxin diluted 1 : 10	..	0	150
Control not immunized but injected with undiluted toxin	..	..	280
Controls without any treatment	..	..	125

In order to exclude possible errors from an enlargement of the spleen by the previous immunization, the mode of the immunization was changed in a following experiment. The detoxicated materials were injected in this test subcutaneously instead of intraperitoneally. Two subcutaneous injections of 0·2 c.c. of toxoid (mixture of nine parts of the detoxicating solution and one part of the toxin) were given. The injections were given in three-day intervals, and the test dose of 0·2 c.c. of the undiluted toxin was given eight days after the last toxoid injection. Three control groups were employed. The first received simultaneously with the toxoid-immunized groups 0·2 c.c. of toxin diluted 1 : 10; the second group was not immunized and received the test dose of 0·2 c.c. undiluted toxin; the third group received no injection whatsoever.

TABLE V.—*Effect of Immunization with Detoxicated Material Given s.c.*

Immunized by toxin treated with—	Concentration of detoxicating substance per cent.	Deaths during immunization.	Average weight of spleen (mg.).
Formalin	0·05	1	120
	0·1	2	100
	0·2	3	90
Permanganate	0·01	0	120
	0·02	0	90
	0·04	0	90
Bisulphite	1·0	0	90
	2·0	0	70
Immunized with toxin diluted 1 : 10	..	0	80
Not immunized, but injected with undiluted toxin	..	..	250
Without any treatment	..	..	90

The experiment represented in Table V shows that by two subcutaneous injections of toxoid or diluted toxin it was possible to immunize against a test dose of 0·2 c.c. of undiluted toxin. In order to be sure that the treated toxins were innocuous, two groups of rats, one weighing 15 g. and the other one 25 g., were injected with toxoids intraperitoneally as well as subcutaneously. The spleen weights are summarized in Table VI.

TABLE VI.—*Toxicity Test of the Toxoids Used in the Immunization Experiment Represented in Table V.*

Material injected.	Mode of injection.	Average spleen weight (mg.) of rats weighing—	
		15 g.	25 g.
Toxoids as in Table V	i.p.	75–100	80–125
	s.c.	80–100	75–110
Toxin undiluted	i.p.	240	250
	s.c.	250	250
Toxin diluted 1 : 10	i.p.	150	150
	s.c.	150	150
Controls untreated	..	85	90

This control experiment shows that the quantities of toxoid employed in the experiment shown in Table V were insufficient to provoke an enlargement of the spleen. On the other hand, the untreated toxin even diluted 1 : 10 sufficed to evoke a well-marked reaction.

CONCLUSIONS.

The labile toxin of *R. prowazeki* described by Kligler and Oleinik (1944) is present in the supernatant fluid of centrifuged as well as in Seitz and Mandler filtrates of yolk-sac cultures. The toxin elicits severe enlargement of the spleen in young rats of 15–25 g. Exposure of the toxin solution to the action of formalin, potassium permanganate or sodium bisulphite of different concentrations for 24 hours in the ice box causes its detoxication, but does not destroy its ability to produce immunity against the effect of the original toxin.

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SPONTANEOUS CRYSTALLIZATION OF A PROTEIN FROM PATHOLOGICAL HUMAN SERUM.

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In previous reports a pathological globulin fraction was described. This fraction was particularly abundant in sera of dogs with kala azar (Stein and Wertheimer, 1942). It was present also in other chronic infectious diseases, especially in endocarditis lenta (Wertheimer and Stein, 1944). The pathological globulin precipitated on cooling to about 4° C., and redissolved on rewarming to 37° C.

In connection with this observation a great number of sera was tested for the presence of this cold-susceptible protein. In one case, to be reported here, the precipitation was conspicuously abundant and rapid, and upon further examination was found to possess a regular crystalline form. This case is reported in the hope that it may induce others to carry out similar examinations in suitable cases.

MATERIAL.

Patient F. B—, aged 62, suffered for 19 years from bronchial asthma. During a stay in hospital in 1943 the following findings were recorded : Bronchial asthma, emphysema, arteriosclerosis generalis, acrocyanosis, unusually strong vascular response to epinephrine. In 1943, sympathectomy of the arteriae femoraliae for torpid ulcers of the foot. During the operation a thickening of the arterial wall was noted, but the ulcers healed quickly. Normal temperature, urine, and blood pressure until the last admission late in 1945, with an attack of hypertensive encephalopathy. An active nephritis with quickly aggravating renal insufficiency set in. One month before death fever of a septic type appeared for the first time. Death in uraemia. The main pathological findings were : Periarteritis nodosa (acute and chronic stage) in myocardium, kidneys, liver, spleen, pancreas and mediastinal fat tissue. Multiple embolism in renal cortex originating from thrombi in foci of arteritis. Acute fibrinous pericarditis. Cardiac hypertrophy. Enlargement of spleen.

THE CRYSTALLINE PROTEIN.

Serum taken during the last two weeks of life became turbid almost immediately after separation by centrifuge. After several minutes a precipitate settled to the bottom, and upon microscopic examination was found to consist of regular crystals (Fig. 1). Crystallization could be almost completely prevented

FIG. 1.—Protein crystals. × 480.

by keeping the temperature of the blood during and after the separation of the serum at 38° C. Cooling to 4° C. enhanced the crystallization. In some sera in which no precipitate appeared even after cooling, crystallization could be induced by diluting the serum with three volumes of water and cooling.

The crystals were found to be insoluble in distilled water, sparsely soluble in ice cold saline (0·9 per cent NaCl) and soluble in saline at 38° C. Crystals washed twice with cold saline and five times with distilled water gave all the protein

reactions, xanthoprotein, biuret, glyoxylic acid test, as well as a positive Mohlisch reaction for sugars. Their solution in warm saline coagulated at 78°–80° C. and could be salted out with ammonium sulphate at 37 per cent saturation. The isoelectric point, tested with McIlvaine's buffer mixture, was found between pH 4·8–5·0. The concentration of the crystals in serum was found to be 270 mg. per cent. Analysis of the other protein constituents gave the following results: Total protein, 6·1 per cent; albumin, 4·1 per cent; total globulin, 2·0 per cent; euglobulin, 0·5 per cent; pseudoglobulin I, 0·75 per cent; pseudoglobulin II, 0·75 per cent; NPN, 145 mg. per cent.

When injected into rabbits in quantities corresponding to 4–5 ml. serum the crystals produced an elevation of body temperature of about 1°–1·5° C. This elevation persisted for one day.

SUMMARY.

The separation of a crystalline protein from the serum of a patient with periarteritis nodosa is described.

We are indebted to Prof. Franco and Dr. Ungar for the pathological details, and to Dr. Rachmilevitz and Dr. R. Shapiro for their aid in the clinical part. This work is aided by a grant from the Dazian Foundation for Medical Research.

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MODIFICATION OF HUMAN RED CELLS BY VIRUS ACTION. I. THE RECEPTOR GRADIENT FOR VIRUS ACTION IN HUMAN RED CELLS.

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In several papers incidental observations have been recorded on the changes induced in red cells which have been subject to the action of viruses of the influenza group. It was shown in 1942 (Burnet, 1942) that cells treated with Newcastle disease virus become resistant to agglutination by that virus but are still agglutinable by the influenza viruses. Further work showed that the viruses could be arranged in a linear series such that cells rendered resistant by treatment with a given virus failed to be agglutinated by virus strains earlier in the series but were still agglutinated by those succeeding it (Burnet, 1945*a*, *b*). The series in abbreviated form is: Mumps, Newcastle disease, most influenza A strains, influenza B, swine influenza.

Recently we have commenced a more intensive study of this phenomenon using human red cells in the belief that increased understanding of some of the problems of human virus infections might be gained thereby. The starting-point for a new approach was the finding that cells treated with virus became agglutinable by human and other sera previously without action on them. Following up this lead it was found that the two first members of the series produced changes in the red cell surface of a wholly distinct category. By suitable treatment one can obtain mumps-treated cells which are specifically agglutinated by mumps antibody to high titre. Cells treated with Newcastle disease virus are agglutinated not only by appropriate animal immune sera, but also by the majority of human sera from cases of infectious mononucleosis. The results of work on these two aspects are presented in separate papers.

In view of the enzyme-like character of the action of influenza viruses on red cells, a survey of a variety of enzyme preparations was made for similar activity. Positive results were obtained with a preparation of *Cl. welchii* toxin type A, the best known enzyme of which is a lecithinase.

The action of bacteria in modifying the agglutinability of human red cells by serum was described by Thomsen (1926) and Friedenrich (1927), and from the latter's description of the action of the enzyme concerned it seemed likely that the effect was similar to those we had obtained by the action of swine influenza virus. Friedenrich (1928) found two groups of bacteria which produced the Thomsen phenomenon of "pan-agglutinability" of human red cells. The first were diphtheroids, the second vibrios, including the cholera vibrio. Using filtrates from a cholera vibrio strain we have shown that changes in the red cells can be produced closely similar to those obtained by treatment with influenza viruses. What may be called the virus-receptors of the red cells are removed in almost the same order, and under appropriate conditions pan-agglutinability is

produced. There is evidence, however, that the two phenomena are not directly correlated, since pan-agglutinability is produced by Friedenrich's strain M (diphtheroid) without loss of virus receptors.

1. *The Receptor Gradient as Shown with Human Erythrocytes.*

The essential characteristics of the receptor gradient can be clearly demonstrated with human red cells of Group O which have the advantage over fowl cells that they are susceptible to O type influenza virus-A strains, and over guinea-pig cells in not being agglutinated by any human sera. The experiment on which Table I is based was carried out on a single batch of washed Group O human cells.

Technique.—Cells were taken in citrate saline, spun down, washed once with normal saline and stored in the refrigerator as packed cells. The experiments were completed within three days. Fresh allantoic fluids infected with the following virus strains were used in treating the cells.

W.S.E.	The classical W.S. strain adapted to chorioallantoic passage, but otherwise almost unaltered in characteristics.
BEL. D.	An A strain isolated in 1942 and characteristic of strains isolated from the Victorian epidemic of that year.
IAN–O IAN–D	O and D phases of a strain isolated in February, 1946, from a sporadic case of influenza A.
SWINE	Shope's strain 15.
N.D.V.	Newcastle disease virus, Victorian strain of Albiston and Gorrie.

In general 10 c.c. of allantoic fluid virus was used to emulsify 0·25 c.c. of packed cells. The tubes were placed in a water bath at 37° for 2 hours, being shaken by hand once or twice during the period. The cells were then centrifuged down and supernatant fluids discarded. Saline was added to give a 2 per cent suspension of cells. These were tested for stability, and where necessary a limited titration of the amount of immune serum necessary to stabilize the cells was carried out (see Section 2). Immune ferret or monkey serum was used, and the necessary amount then added to the rest of the cell suspension. After 30 minutes at room temperature to allow equilibration the tubes were again spun and the cells finally emulsified in saline.

These cells were then tested, along with cells similarly treated except that saline replaced the allantoic fluid virus, against a single dilution of each of a number of virus samples, including those used in treating the cells and the following additional ones:

Mumps.—Strain SOM from Dr. W. I. B. Beveridge.
MELB.—Standard strain isolated in 1935.
LEE–B.—Standard B strain.
MIL-B and HUT-B.—Two B strains isolated in Melbourne in 1945.
HOL-O, BEL-O } Stocks of glycerolated amniotic fluid containing O phase virus of these A strains prepared in 1943.
MIL-A O, MIL-A D } O and D phase amniotic fluid from a recently isolated strain of influenza A.
HOL-D.—Concentrated D phase virus from strain tested in O phase above.

TABLE I.—*The Receptor Gradient in Human Cells.*

Cells treated with—	Tested with 5–10 agglutinating doses of—														Saline control.
	1 MU.	2 NDV.	3 MEL.	4 MIL-AO.	5 WS.	6 BEL-D.	7 IAN. O.	8 MIL AD.	9 LEE	10 BEL D.	11 IAN D.	12 SWINE	13 HUT B.	14 MIL B.	
Saline	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
2 N.D.V.	−	−	±	..	+	..	+	..	+	+	+	+	+	+	−
5 W.S.E.	−	−	−	−	−	±	+	+	+	+	+	+	+	+	−
7 IAN-O	−	−	−	−	−	−	−	±	+	+	+	+	+	+	−
10 BEL-D	−	−	−	−	−	−	−	−	−	−	±	+	+	+	−
12 Swine 1 hr.	−	−	−	..	−	..	−	..	−	−	−	+	+	+	−
,, 5 hr.	−	−	−	..	−	..	−	..	−	−	−	−	−	−	−

\+ Agglutination essentially equivalent to that with normal cells.
± Agglutination less complete than control.
− No agglutination.

The results in Table I show an almost completely symmetrical arrangement; the virus strains can be placed in linear order such that when a cell is rendered insusceptible by the action of any given virus V it is not agglutinated by viruses which precede V in the series, but is agglutinated by all those which follow it.

Further elaboration of the table in regard to strains not differentiated by the experiment shown concerns the relative position of mumps and Newcastle disease virus at one end of the series and of swine influenza and the two recently isolated B strains at the other. Cells rendered resistant to agglutination by treatment with mumps virus are consistently susceptible to agglutination by all the other viruses of the series. Very little difference is shown between Swine influenza virus and the recent B strains typified by MIL-B. With fowl cells SWI appeared to be more active, but with human cells rendered resistant to all but this group MIL-B shows a higher titre than SWI, so is placed at the end of the series.

2. *The Development of Pan-agglutinability by Virus Treated Cells.*

In earlier experiments it was concluded that Swine virus could not be eluted from fowl cells either spontaneously or by the use of immune sera (Burnet, 1945*a*). The second part of this conclusion was erroneous, the agglutination observed after treatment of the unstable cells with serum being due not to residual virus, but to the serum. The fact that the serum had no action on untreated cells was at the time regarded as indicating that it would not agglutinate the treated cells. The inadequacy of this control can be seen in the following experiment:

To 10 ml. of Swine influenza allantoic fluid virus was added 0·25 c.c. of packed human cells. After standing at 37° for ½, 1, 4 and 6 hours, samples were removed, the cells centrifuged and emulsified in saline to give 2 per cent suspensions. These unstable suspensions were then added to a series of dilutions of an immune ferret serum F217 (Swine), and after contact for 10 minutes the tubes were reshaken and the cells allowed to sediment in the usual fashion.

TABLE II.—*Development of Serum Agglutinability by Treatment with Swine Influenza Virus.*

Treatment of cells.	Immune serum dilutions. 10.	20.	40.	80.	160.	320.	640.	Saline control.
Nil	—	—	—	—	—	—	—	—
SW.V. 30 min.	+	±	—	—	—	+	++	++
,, 1 hr.	+	±	—	—	—	++	++	++
,, 4 hr.	++	++	+	—	—	+	++	++
,, 6 hr.	++	++	++	++	+	+	++	++

It will be seen that the cells rapidly become susceptible to agglutination by the lower dilutions of serum, and that this susceptibility increases as the action of the virus is prolonged. Provided a reasonably short period of contact is used, stable cells can be obtained by treatment with immune serum diluted beyond the agglutinating level.

It was readily shown that the agglutination by the lower serum dilutions was not related to the antibody present, but was given by almost all mammalian sera.

Very few human sera failed to agglutinate cells treated with swine influenza virus to a titre of 160. Guinea-pig and rabbit sera showed lower titres around 10–40 for treated cells. Guinea-pig cells which untreated are not agglutinated by such sera were used for these tests.

The titre obtained with normal human sera is roughly proportional to the position in the receptor gradient of the virus used to prepare the cells. Fig. 1 shows the distribution of titres in a group of human serum samples when these were tested against cells treated with Swine influenza virus, while Fig. 1 in the

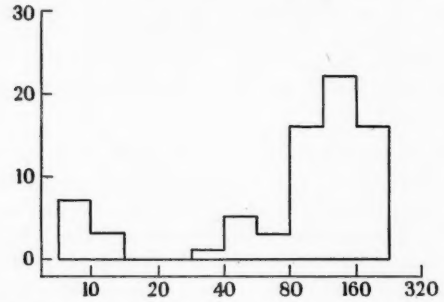

FIG. 1.—Distribution of agglutinin titres of 73 normal human sera against human cells treated with swine influenza virus.

next paper of this series shows similar results with cells treated with N.D.V. Table III gives the titres for five sera tested with cells treated with WS and MIL-B as well as the other two viruses.

TABLE III.—*Serum Agglutination of Cells Treated with Different Viruses.*

	Titre with cells treated with—			
Serum.	NDV.	WS.	Swine.	MIL-B.
1	20	15	100	140
2	8	10	80	400
3	25	30	120	400
4	25	20	80	200
5	50	40	120	400

There is some variation from one batch of cells to another, but the table is representative of the results.

A small proportion of sera from influenza patients shows a rise in titre between the acute and convalescent serum samples. In the table sera 2–3 and 4–5 represent two such pairs. It will be seen that the rise is shown (with one exception) with each type of treated cells. A good deal of investigation has been done on this point, and on the related finding that animals inoculated intravenously with influenza viruses show irregularly a heightened titre against treated cells. It was concluded that such changes in the serum were nonspecific in character and no experimental details will be given.

3. *Destruction of Virus Receptors by Bacterial Products.*

The first suggestion that receptors could be removed from red cells by agents other than the viruses concerned was obtained in experiments made by one of us (J. F. McC.), using a partially purified preparation of *Cl. welchii* toxin Type A. Since the most active enzyme known to be present in such material is a lecithinase, attention was directed toward other bacteria known to possess lecithinase activity. The cholera vibrio seemed particularly suitable for study since it was known to have high lecithin-splitting activity, and had also been shown by Friedenrich (1928) to be able to induce Thomsen's phenomenon of pan-agglutinability in human red cells. It was found that filtrates made from 20-hour broth cultures of *V. cholerae* strain 4Z were also capable of removing the virus receptors from human red cells.

A. *Action of* Cl. welchii *toxin on red cells.*

A dried preparation of *Cl. welchii* toxin Type A was kindly provided by Dr. A. W. Turner. This had been prepared by precipitation with saturated ammonium sulphate. A stock 1 : 500 solution was prepared in saline, and the experiments were carried out with a further 10- or 20-fold dilution of this. Experiments were first made with small amounts of added calcium, but this resulted in gross haemolysis without evidence of receptor destruction. When normal saline only was used and the reaction carried out at 45° with a 2 per cent suspension of group O human cells no haemolysis occurred, and the results shown in Table IV were obtained.

TABLE IV.—*Progressive Action of* Cl. welchii *Toxin on Human Red Cells.*

Time of action.	Agglutination by viruses—						
	MU.	NDV.	MM.	WS.	LEE.	BEL.	SWI.
½ hour	++	++	++	++	++	++	++
1½ hours	−	±	+	±±	++	++	++
3 ,,	−	−	−	−	+	++	++
6 ,,	−	−	−	−	−	±±	±±
6 hour control	±±	++	±±	++	++	++	++

2 per cent cells treated 1 : 10,000 toxin in normal saline at 45° C.

B. *Action of filtrates from* V. cholerae *in removing virus receptors from human red cells.*

Filtrates were prepared from a 20-hour broth culture of *V. cholerae* strain 4Z by preliminary centrifugation followed by passage through a gradocol membrane of A.P.D. 750 mμ. Serial dilutions of the filtrate were made in normal saline in 1 ml. amounts, giving a range of doubling dilutions from 1 to 1 : 128. To each tube 0·05 ml. of packed washed Group O human red cells was added, and the series maintained at 35–37° in an incubator for 2½ hours. The tubes were then centrifuged and the supernatant fluid discarded. The cells were made up to 2 per cent suspensions by the addition of 2·5 ml. of saline, and each suspension tested for

susceptibility to agglutination by our standard series of viruses. The virus dilutions were adjusted to contain 5–10 agglutinating doses for normal human cells.

With the same filtrate a 1 : 4 dilution was prepared, and 4 ml. of this treated for one hour at 0° C. with 0·25 ml. of packed human cells. The cells were then deposited in the centrifuge and the supernatant fluid preserved (" cold supernatant " C.S.). The cells were washed with 10 ml. of cold saline and re-emulsified in 4 ml. of saline before being placed in a water bath at 37° for three hours. They were then centrifuged. The supernatant fluid (" Eluate " EL) was preserved and the cells made up to 5 ml. for testing. Both C.S. and EL were now titrated for their power to modify human cell receptors by the same method as was used

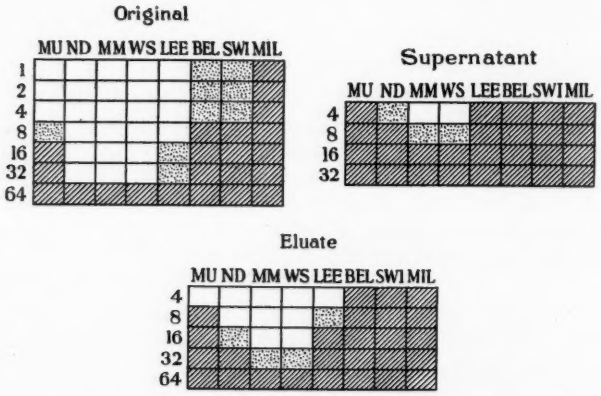

FIG. 2.—Agglutination by the standard series of viruses on cells treated : A. With original filtrate from culture of cholera vibrio 4Z. B. Supernatant fluid after absorption of filtrate in the cold. C. Eluate from the cells used to absorb in B. The dilution of the material used is shown at the side of each block. Shaded areas equal complete agglutination ; stippled areas, partial agglutination ; clear areas, no agglutination.

for the original filtrate, the first dilution in each case corresponding to 1 : 4 of the original material.

Fig. 2 shows graphically the results obtained.

It will be seen that susceptibility to agglutination by different viruses is progressively lost in almost the same sequence as results from virus action. The only outstanding discrepancy is the undue retention of susceptibility to agglutination by mumps virus.

These experiments are representative of a number made with cultures of *V. cholerae* 4Z and Inaba and a strain of *V. paracholerae*. Unfiltered young broth cultures of all three strains showed the phenomenon clearly. With older cultures difficulties were experienced owing to the power of the cultures to agglutinate human cells. When the treated cells were only moderately unstable it was found possible to make satisfactory tests by adding about 0·5 per cent of sodium citrate. High activity was observed in the supernatant fluid from culture of 4Z washed off with saline from an agar slope. The addition of 50 units of

penicillin as a bacteriostatic agent to dilutions of this fluid did not interfere with the process of receptor removal. Elution of the active agent from washed cells was also demonstrated with this material.

DISCUSSION.

The ability of two different types of bacterial products to change the surface character of human red cells so that they become progressively less sensitive to agglutination by viruses is clearly a function of one or more enzymes in the material. The possibility that lecithinases are concerned is suggested by the fact that active enzymes of this character are produced by both *Cl. welchii* and the cholera vibrio, but no specific evidence on the point is available. The changes produced are very closely similar to but not identical with those which are produced by the action of successively more active viruses on human red cells, and the conclusion becomes almost irresistible that the viruses exert their effect by similar enzymes or enzymatically active groupings on the surface of the virus particle.

In the course of this work two differences in the activity of bacterial enzymes on the one hand and the action of influenza viruses on the other have been observed: (1) Mumps virus receptors are more readily removed by virus action than by vibrio filtrates, and there is a less evident but probably significant difference in the same direction with Newcastle disease virus receptors. (2) With vibrio filtrates removal of virus receptors may be almost complete, with very little development of serum agglutinability by the cells. Unfiltered cultures have usually produced both effects, while older cultures have rendered the cells permanently unstable and therefore unsuitable for test. Conversely the Friedenrich M strain has so far produced serum agglutinability without loss of virus receptors. The possibility that more than one cell surface component and more than one enzyme are concerned in these reactions must, therefore, be kept in mind.

Further work will be directed toward the characterization of the enzyme or enzymes responsible for these effects. It may be possible to utilize the elution phenomenon described with cholera vibrio filtrates for the isolation of the enzyme in a fashion similar to Francis and Salk's (1942) method for the concentration of influenza virus. In any case the clear-cut indication of reaction which is available in the tests for the presence or absence of virus receptors should make the task of characterizing the enzyme responsible a relatively straightforward matter.

Should it develop that similar enzymes are an intrinsic part of viruses of the mumps-influenza group, two important possibilities arise: (1) that these enzymes play an essential part in the initial invasion of susceptible cells as well as in adsorption to erythrocytes, and (2) that the nature of the enzymes may open up an opportunity for chemotherapeutic studies.

SUMMARY.

A " receptor gradient " in human red cells is defined for viruses of the mumps-influenza group.

Cells which have been treated by influenza viruses in addition to losing their agglutinability by some or all of the viruses of the group develop a susceptibility

to serum agglutination analogous to the "pan-agglutinability" described by Thomsen.

Virus receptors can be removed in almost the order of the receptor gradient by enzymes produced by *Cl. welchii* and *V. cholerae*.

The significance of this finding is discussed.

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MODIFICATION OF HUMAN RED CELLS BY VIRUS ACTION. II. AGGLUTINATION OF MODIFIED HUMAN RED CELLS BY SERA FROM CASES OF INFECTIOUS MONONUCLEOSIS.

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IN the course of a comprehensive study of the changes induced in human erythrocytes by viruses of the influenza-mumps group considerable use was made of cells treated with the virus of Newcastle disease of fowls (N.D.V.). An observation that a serum from a patient with infectious mononucleosis agglutinated cells treated with N.D.V. to a titre of 15,000 naturally aroused a much increased interest in this virus and stimulated the present investigation. The observation arose from an investigation of the power of most human sera to agglutinate red cells from which influenza virus had been eluted. This work will be described elsewhere, but it may be said that human cells treated with viruses of this group undergo a surface change which results in their becoming agglutinable by a variety of normal sera. In general, the titre given by a normal serum is low for viruses placed early in the linear series recently described (Burnet, 1945*b*), higher for those later in the series. Cells treated with N.D.V. and with Swine influenza virus were taken as representing one of the earliest and the last of the series and were used to test a considerable number of human sera. In view of the Paul-Bunnell test it was natural that sera from infectious mononucleosis should be included amongst those tested.

Our work to date indicates that the agglutination of N.D.V.-treated human cells by infectious mononucleosis sera is not of the same type as the "normal serum" agglutination of cells treated by viruses of the group in general, but is due to the existence of a specific antigen derived from the virus and adsorbed on to the red cell surface.

Technique.

The N.D.V. strain used was of Victorian origin; the circumstances of the outbreak concerned have been described by Albiston and Gorrie (1942). It has been maintained by chick embryo passage, mainly by the amniotic route.

The methods of chick embryo inoculation and of haemagglutinin tests were those in general use in this laboratory and have been frequently described. Human red cells were obtained from Group O donors, washed first in normal saline then in " calcium saline " (0·85 per cent NaCl, 0·024 per cent $CaCl_2$), and were made up to the required dilution in calcium saline. Unless specifically stated " saline " throughout the paper refers to calcium saline.

The Action of Newcastle Disease Virus on Human Red Cells.

In previous papers the general character of the haemagglutination produced by N.D.V. with fowl cells has been fully described. In comparison with influenza virus agglutination the salient features are (1) a less active clearing of the cell suspension, the agglutination being best demonstrated by the pattern of the deposited cells (Lush, 1943), (2) a rapid elution of virus and complete subsequent stabilization of the cells (Burnet, 1942), (3) treated cells are insensitive to the agglutinating action of N.D.V. (and mumps virus), but show almost unaltered susceptibility to agglutination by influenza viruses (Burnet, 1945*a*).

Human cells are slightly less readily agglutinated than fowl cells, but show all three features as clearly as the latter. Elution and stabilization can be very easily demonstrated if human cells are added to serial dilutions of virus and the mixtures kept at 37°. With 0·5 c.c. total volume in 10 mm. diameter tubes deposition is practically complete in one hour. If the rack is shaken after the reading is made and again allowed to stand, it will be found that by the end of the second hour all tubes containing more than about 40 agglutinating doses of virus show stabilized unagglutinated cells. The end-point normally extends slightly between first and second reading.

TABLE I.—*Agglutination of Human Red Cells by N.D.V. (Amniotic Fluid) at 37°.*

	Virus dilution.							
	10.	20.	40.	80.	160.	320.	640.	1280.
1st reading .	±	++	++	++	++	+	tr.	—
2nd reading .	—	—	+	++	++	++	+	—

When cells treated with virus and stabilized are tested with an appropriate series of viruses they show the behaviour exemplified in Table II. Each virus was titrated on normal human cells and cells treated with N.D.V. The figure shown is the approximate percentage of the normal titre shown with the treated cells.

The results with MELB and WS vary with the conditions, but the agglutinability of human cells for these two strains is always greatly reduced by treatment with N.D.V. Fowl cells similarly treated show less reduction in agglutinability by these two influenza viruses.

TABLE II.—*Percentage Titre of Viruses Tested Against Human Cells Treated with Newcastle Disease Virus.*

Virus.		Per cent titre.
Mumps		0
N.D.V.		0
Influenza viruses	Melb. M	1
	W.S.	2
	LEE	50
	BEL	100
	MIL-B	100
	SWI	100

The influenza virus strains have all been previously described; Melb., W.S. and BEL are A strains, LEE and MIL-B are type B, and SWI is Shope's Swine influenza strain 15.

A broader discussion of the nature of these induced changes in red cell receptors will be given in another communication. They are mentioned here to establish the close relationship of N.D.V. in this respect at least to mumps and influenza viruses, and to support the view to be put forward below that some of the changes induced in red cells by N.D.V. are of the same general character as those produced by influenza viruses lower in the series.

The Agglutination of Human Cells Treated with N.D.V. by Human and Animal Sera.

Fowls can be immunized against N.D.V., which is normally highly lethal to the species, by the injection of formalinized virus. When immunization is followed by a challenge dose of active virus the birds develop high titre antibody. The antibody is most readily demonstrated by using a Hirst type inhibition of haemagglutination test. Essentially similar antibody can be produced by the intravenous or intracardiac inoculation of active virus in rabbits or guinea-pigs. Table III gives the results obtained in comparative tests with sera from two

TABLE III.—*Agglutination of Treated Cells with Specific Immune Sera.*

Sera.			Anti-haemagglutinin titre.	Agglutination of treated cells.	
				NDV-G	NDV.H
Guinea-pig	176	I	10	10	15
		II	70	480	350
,,	178	I	10	10	5
		II	70	560	800
Rabbit	125	I	10	..	60
		II	400	..	600
,,	127	I	10	..	80
		II	240	..	800
Fowl	1	II	200	..	320
,,	2	II	240	..	560

NDV.H Human cells treated with virus N.D.V.-G. Guinea-pig cells similarly treated.

individuals from each species, both the haemagglutinin-inhibition and treated cell agglutination methods being used.

Since guinea-pig and fowl cells are agglutinated by N.D.V., it is possible that the antibody from these species causing agglutination of the treated cells is due to immunization of the animal with its own altered cells. This possibility is excluded for the rabbit since rabbit cells are unaffected by N.D.V., and it is reasonable to assume that in each instance the agglutination of N.D.V.-treated cells is due to a specific antibody against a virus product in the serum. There is nothing incompatible with the simple view that the antibody is the same as that responsible for inhibition of haemagglutination in the conventional set-up.

It should be mentioned, however, that cells treated with N.D.V. are agglutinated to lower but still considerable titres by serum from animals inoculated with other viruses, e.g. swine influenza virus. As an example we may cite the serum of guinea-pig 167, which received two intracardiac inoculations of swine influenza virus (Table IV).

TABLE IV.—*Agglutination of N.D.V.-treated Cells with Heterologous Guinea-pig Serum.*

	Cells treated N.D.V.			Cells treated SWI V.
	Fowl.	Guinea-pig.	Human.	Guinea-pig.
Pre-inoculation serum .	<10	<10	<10 .	<10
After Swine virus 1 . .	80	100	60 .	60

About 70 normal human sera were tested, mostly consisting of samples obtained in connection with influenza investigations. Nearly all showed some power to agglutinate human cells treated with N.D.V., but the titres were all below 1 : 120 and were distributed as shown in Fig. 1. No significant variations were evident in pairs of acute and convalescent sera from patients with influenza.

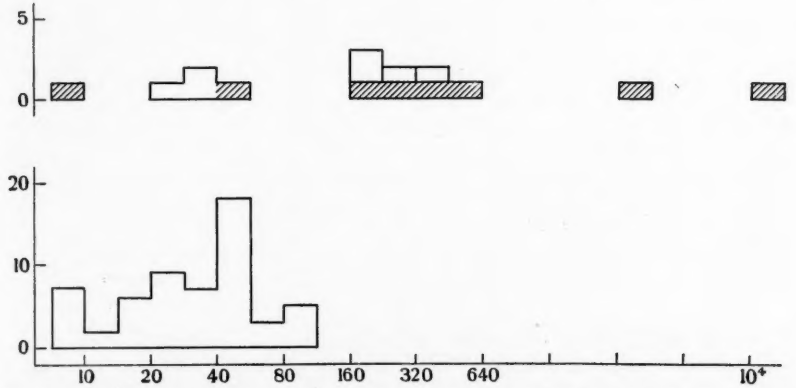

FIG. 1.—Distribution of agglutinin titres against cells treated with N.D.V. of sera from 15 cases of infectious mononucleosis (above) and from 57 normal persons (below). Shaded blocks indicate a positive Paul-Bunnell reaction.

The second serum obtained from patients with infectious mononucleosis was the serum LOC already mentioned. This serum showed a Paul-Bunnell titre with sheep cells of 400, it agglutinated human red cells treated with swine influenza virus to 1 : 120 (a low normal figure), but cells treated with N.D.V. were agglutinated to a titre of 15,000. In view of the interest of this serological reaction a brief history of this patient is given.

LOC. Male, aged 20, was admitted to the Royal Melbourne Hospital as a possible case of typhoid fever with complaints of headache, malaise, sore throat and abdominal pain. On examination axillary and posterior cervical lymph nodes were enlarged. On 31.i.46 his blood showed a leucocyte count of 7700 with 29 per cent neutrophils and the remainder mononuclear cells including atypical forms. A week later the leucocyte count was 16,500 with 45 per cent small and 34 per cent large lymphocytes, 8 per cent atypical cells and only 13 per cent of polymorphonuclear leucocytes. The Paul-Bunnell test on 6.ii.46 gave a titre of 1 : 256. The serum used in our experiments was obtained on 15.ii.46, approximately a month after onset of the illness and a week after the patient had left hospital apparently well. A further specimen LOC II was obtained on 25.iv.46.

During April, 1946, a number of cases of infectious mononucleosis were diagnosed at military and civil hospitals in the Melbourne region and we received blood from 15 possible cases. Those in which the diagnosis was reasonably certain from the blood picture and clinical history are shown in Table V.

TABLE V.—*Serological Reactions of Persons Diagnosed as Suffering from Infectious Mononucleosis.*

Patient.	Approximate time since onset (weeks).	Paul-Bunnell titre.	N.D.V.-human cell agglutination.
SET	3	400	320
LOC I	4	400	15,000
,, II	14	160	400
COA	3	2000	50
LAN I	1	200	<10
,, II	4	640	50
BYR	5	200	240
FEW	5	300	200
WAS	1	1280	500
WAT	4	50	30
GEO	2	40	320
ANG	25	<40	200
MAP	..	40	25
MIL	4	120	40
ROS	1	80	18

It will be seen that of the seven individuals with a diagnostic Paul-Bunnell titre, five showed agglutination of N.D.V.-treated cells to a higher titre than was observed with any normal serum (i.e. >120). In the group of six with a Paul-Bunnell titre below 160 two agglutinated the N.D.V. cells to a significantly high level.

The results may be summarized as showing (1) that cells treated with N.D.V. are agglutinated to low titres by almost all normal human sera. This agglutination is of the same general type as that given with cells treated by any of the influenza viruses. Evidence has been given in the previous paper that this change produced by all viruses of the mumps-influenza group to varying degree is probably related to the " panagglutinability " described by Thomsen (1926) and Friedenrich (1927) as resulting from the action of certain bacteria on human red cells.

(2) That sera from more than half the cases of infectious mononucleosis available for study contain an antibody of different character which acts only on cells treated with N.D.V. and not on cells treated with other viruses of the group.

A reasonable amount of the serum LOC I was available, and this has been used in a study of the nature of the change in human red cells rendering them agglutinable to high titre by this serum.

The Cell-sensitizing Agent in N.D.V. Preparations.

Unpublished work on the changed agglutinability of red cells treated with influenza viruses indicates strongly that this is due to a modification induced in the surface of the cell by adsorption and elution of the virus, the " antigen " concerned being a modified cell constituent, not a direct product of the virus.

The agent concerned in the agglutination by LOC serum was obviously of a different character, and the two major alternatives as to its nature were clearly, (*a*) that another type of modification had been induced in the cell surface by the virus, or (*b*) that a virus product adsorbed to the cell and not eluted with the virus was responsible.

An immediate finding in favour of the second alternative was that fowl, guinea-pig and human cells similarly treated with N.D.V. were all agglutinated to similar high titres by serum LOC I.

In order to differentiate formally between the two alternatives, experiments were planned by which active virus preparations might be obtained free of the hypothetical cell-sensitizing agent or by which the latter might be obtained free of the virus.

The first type of experiment was designed to examine two sorts of evidence which would favour the existence of a substance (other than the active virus particles) which was responsible for sensitizing the cells. In the first place, if cells are treated with falling concentrations of a virus preparation at 37° complete elution and stabilization takes progressively longer with increasing dilution, but if the effect is due only to the action of the virus, it should be demonstrable to the highest dilution producing complete elution and stabilization. Secondly, if the supernatant fluid containing eluted virus is treated successively with fresh cells, any unelutable sensitizing material should be rapidly removed while the virus titre will fall much more gradually.

The following is an experiment of this type : Serial dilutions were prepared in saline of a high titre amniotic fluid virus in volumes of 1 ml., the range of dilutions being from 1 : 10 to 1 : 160. To these 0·05 ml. of a 20 per cent suspension of human red cells was added. The tubes were placed in a 37° incubator and examined and reshaken at hourly intervals. In this way the progress of stabilization of the cells could be observed. This appeared first in the tubes

with the highest concentration of virus and was evident in the final dilution after 4 hours. When the pattern of deposited cells showed complete stabilization the tube was removed, reshaken and centrifuged. The supernatant fluid was removed and preserved, the cells were resuspended in 1 ml. of saline and this suspension tested with 1 : 1000 serum LOC for specific agglutination. When the supernatant fluids were all available a fresh amount of red cells (0·05 ml. of 20 per cent suspension) was added and the process repeated.

TABLE VI.—*Absorption of C.S.A. by Red Cells and its Differentiation from Virus.*

Cells treated with—	Initial virus dilutions. 10.	20.	40.	80.	160.	Direct haemagglutinin titres.	
Original virus	++	++	++	+±	+	Original virus . .	400
1st supernatant	++	+±	±	−	−	3rd supernatant (1 : 10)	280
2nd supernatant	±	−	..	..	..	(1 : 20)	200

In the first section of the table is shown the agglutination of treated cells with 1 : 1000 dilution of serum LOC. The haemagglutinin titres are those obtained with human cells at 37°. For further details see text.

This experiment provides a clear differentiation between the agent responsible for rendering the cells agglutinable by the specific antibody in serum LOC and the virus. With each addition of cells the power to sensitize cells diminishes sharply, but the third supernatant fluid still contains about 50 per cent of the original virus as judged by its direct haemagglutinin activity.

A more direct approach to the same problem of the differentiation of virus and sensitizing agent can be made by eluting the virus from washed cells, and showing that this has no power to sensitize the cells.

One ml. of a high titre amniotic fluid virus was diluted with 2 ml. of saline and 0·1 ml. of packed human cells added. This was kept at 0° for half an hour, centrifuged, and the agglutinated cells washed with cold saline. The cells were then suspended in 1 ml. saline and left for 1 hour at 37° for elution to occur. The eluate, which contained a moderate amount of haemoglobin, had a titre of 80 with human cells, approximately half the original titre of the fluid.

Dilutions of 2, 4, etc., of the eluate were treated each with 0·01 ml. of cells to 0·5 ml. of diluted virus at 37°. After 4 hours stabilization of the first three dilutions had occurred. The cells were spun down, resuspended in saline and tested with serum LOC. No agglutination whatever was observed.

Attempts to prepare material containing the sensitizing agent in the absence of the virus have been limited owing to the absence of any suitable high speed centrifuge. Seitz filtrates of infected allantoic fluid contain no virus detectable by haemagglutination and have no sensitizing effect on human cells.

DISCUSSION.

The use of particulate suspensions of bacteria or collodion particles sensitized by adsorption of soluble antigens or viruses so as to be agglutinable by the corresponding antisera has been described by Goodner (1941) and by Roberts

and Jones (1941). Neither of these tests seems to have been adopted for systematic serological work, and one gathers the impression that the conditions for the production of suitably sensitized suspensions need to be very critically controlled.

The present observations are of some general interest as representing the first utilization of red cell agglutination for demonstrating a virus antigen-antibody reaction. Work at present in progress indicates that a similar red cell sensitizing antigen can be obtained from mumps virus preparations. The red cell has obvious advantages over most other particulate "carriers" for such purposes and the method may find some applications in clinical serology.

The agglutination of cells sensitized with a Newcastle disease virus antigen by a majority of sera from cases of infectious mononucleosis may have one of three explanations:

1. The etiological agent as yet unknown may be a virus antigenically related to Newcastle disease virus and, therefore, probably resembling it in other respects. Newcastle disease in fowls is a highly infectious, highly fatal disease that is enzootic in Java and other regions in the Far East. What appears to be another strain of the virus is, however, responsible for a disease of fowls in California of very different clinical character. It normally produces only a small mortality, and from the combination of respiratory and paralytic symptoms observed has been named by Beach (1945) "pneumoencephalitis of chickens." It is not inconceivable that another strain still might be responsible for a human disease spread by the respiratory route.

2. The relationship between the antibody and the sensitized red cells may be a specifically immunological one, but be due essentially to an accidental resemblance of antigenic structure such as is found between the typhus rickettsia and *Proteus* X 19 or between pneumococcus type II and some vegetable gums.

3. There may be no strictly immunological significance whatever in the finding of these agglutinins. A labile globulin may be produced in the course of infectious mononucleosis which by some accident of molecular configuration reacts strongly with the N.D.V. antigen as well as with sheep cell antigens. The reactivity of the globulin on this view is not a manifestation of specific immunological modification in response to a micro-organismal antigen.

Until the etiological agent of infectious mononucleosis is determined it appears unlikely that an experimental choice between these alternatives will be available. The main value of the present observations may well be to act as a stimulus towards further attempts to establish the etiology of the disease. The working hypothesis that the disease is due to a virus in some ways resembling that of Newcastle disease of fowls at least suggests methods by which the problem might be attacked.

SUMMARY.

The virus of Newcastle disease of fowls (N.D.V.) agglutinates human cells and is eluted therefrom in the same fashion as influenza viruses.

Cells treated with amniotic or allantoic fluid preparations of N.D.V. develop a new antigenic character, which allows them to be agglutinated to high titre either by experimental N.D.V. immune serum or by most sera from recent cases of infectious mononucleosis in man.

The changed character of the cells is due to the adsorption to their surface of an agent other than the virus produced during the growth of N.D.V. in chick embryo cells.

The possible significance of this agglutination reaction as a lead to the etiology of infectious mononucleosis is discussed.

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MODIFICATION OF HUMAN RED CELLS BY VIRUS ACTION. III. A SENSITIVE TEST FOR MUMPS ANTIBODY IN HUMAN SERUM BY THE AGGLUTINATION OF HUMAN RED CELLS COATED WITH A VIRUS ANTIGEN.

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IN the course of studies on the surface changes induced in red cells by the action of haemagglutinating viruses, human red cells were treated with mumps virus preparations. With allantoic fluids of relatively low titre what we regarded as " normal " reactions were obtained. The cells adsorbed and were agglutinated by the virus ; subsequently most of the virus was eluted, leaving still relatively unstable cells. When these were washed they could be stabilized by the addition of a small amount of a human immune serum. Tests of these stabilized cells showed that they had lost their susceptibility to agglutination by mumps virus, but were still susceptible to all the other viruses of the same haemagglutinative type.

When amniotic fluid virus was used, however, quite different behaviour was observed. The partially stabilized cells after the action of the virus were not further stabilized by immune serum in low concentration at room temperature, but appeared to be more firmly agglutinated. Following up this lead it was found that such cells were stable at 37° in the sense of settling to a definite central button at the bottom of the tube, but were agglutinated to high titre with serum from convalescent mumps patients. This test appears to be much more

sensitive than the conventional titration of antibody by its power to *inhibit* agglutination of red cells by the virus.

When higher titre allantoic fluid became available, using a strain received from Dr. Enders, it was found that similarly agglutinable cells could be obtained by the use of dilute virus.

Technique.

Amniotic fluid virus was obtained through the kindness of Dr. W. I. B. Beveridge. It was derived in part from embryos inoculated into the yolk sac at 7 days' incubation and harvested after 7 days' further incubation at 35° C. and in part from 10-day embryos inoculated directly into the amniotic cavity. The titre of the fluids varied from 60 to 200 by direct agglutination of fowl or human red cells.

The treated red cells were prepared by adding 0·2 ml. of packed washed human cells of Group 0 to 5 ml. of a mixture of equal parts of infected amniotic fluid and saline. The tube was placed in a water bath at 37° for 3 hours and left overnight in the refrigerator. The following morning the cells were re-emulsified, centrifuged, and the supernatant fluid discarded. The cells were made up to 2 per cent in saline and tested at 37° for stability and for agglutinability by a known active serum.

With allantoic fluid virus produced from a strain adapted to the allantoic cavity by Enders, suitable agglutinating suspensions can also be prepared, but to date we have had some difficulty in avoiding spontaneous agglutination. This can be overcome by empirical manipulations, and it is probably only a matter of detailed investigation to provide consistently satisfactory suspensions.

The following technique applied to high titre allantoic fluid has proved satisfactory: The allantoic fluid is diluted 1 : 10 in saline and to 10 ml. volumes 0·125 ml. of packed group O cells added. The tubes are placed in a water bath at 37° and left for 2½ hours. They are then centrifuged and the supernatant fluid discarded. The cells are made up to 0·5 per cent suspension in calcium saline, and tested for stability in saline and agglutinability with a known positive serum at a relatively high dilution. We have found that if the cells are left overnight in a refrigerator they tend to show spontaneous agglutination in saline. This can be overcome by holding the cells at 37° for an hour before setting up the tests.

The use of dilute allantoic fluid for sensitizing the cells has been adopted because of the occasional appearance of a secondary agglutination in cells apparently stabilized by relatively concentrated fluid and the failure to obtain satisfactorily stable saline controls. We have not obtained satisfactory preparations with allantoic fluids of titre below 100.

The actual tests were made with 0·5 per cent suspensions of treated cells partly to economize reagents but also because the results were, on the whole, more easily read than those obtained with more concentrated cell suspensions. Doubling dilutions of serum over the required range were made in saline and an equal volume (0·25 ml.) of treated cell suspension added. All saline contained the usual 0·024 per cent $CaCl_2$ in 0·85 per cent NaCl. Readings were made after 2 hours in a 37° incubator, according to the pattern of the deposited cells. The appearances were precisely similar to those of the Salk test, and well marked partial agglutination was taken as the end-point.

The sera tested were obtained from Dr. Beveridge and have been referred to in Beveridge and Lind (1946). They had been stored in the refrigerator for some months.

TABLE I.—*Typical Protocols of Mumps Agglutination Reactions.*

	10	20	40	80	100	200	400	800	1000	2000	4000	8000
B. No history of mumps	−	−	−	−	−							
H. Mumps 4 years previously	++	++	++	+	+	−	−	−				
S. I. Acute stage mumps	±	−	−	−	−	−	−	−				
S. II. Convalescent	−	−	±	++	++	++	++	++	++	++	++	+

++ Complete "shield" agglutination; +, ±, minor degrees of agglutination.

Results.

Table I shows the characteristic types of agglutination observed with sera from persons with known histories of mumps. The existence of a prozone is invariable with high titre immune sera, and all sera should be tested to a dilution above 100 before being recorded as negative.

TABLE II.—*Comparison of Treated-cell Agglutination (T.C.A.) with Haemagglutinin Inhibition (H.I.) Titres of Sera from Mumps Cases.*

Mumps cases.	T.C.A.	H.I.
STO II	3200	640
SOM I	<10	20
,, II	8000	295
FOO I	200	20
,, II	1600	100
Immunized subjects.		
MK I–II	60–400	15–65
PL I–II	<10–320	10–30
McA I–II	15–240	<10–10
LH I–II	80–40	15–40
FB I–II	<10–10	<10–10

Table II gives a brief comparison of results obtained by the present technique with those obtained by conventional haemagglutinin-inhibition methods.

The chief feature of the results is the wide range between normal serum and immune serum value which is always a desirable feature in any serological reaction.

These results are only of a preliminary nature, but establish the practicability and potentialities of the method. Further studies of its use in epidemiological studies of mumps are being undertaken.

The Nature of the Reaction.

The problem immediately presents itself as to the nature of the antigen concerned in this reaction. Is it the mumps virus itself attached to the red cell

surface, a breakdown product of the virus similarly adsorbed, or does it represent an antigen derived from the red cell surface by the action of the virus ? From Table I it is evident that the antibody concerned may result either from infection with mumps virus or from artificial immunization with inactivated allantoic fluid virus. This makes it highly probable that one of the first two alternatives obtains, i.e. that the agent sensitizing the cells is the virus or an antigenically active derivative of the virus. It is hardly conceivable that inactive virus given subcutaneously could so modify the surface of a sufficient number of red cells that these would then act as an effective antigenic stimulus in regard to such modification.

In the light of the results obtained with Newcastle disease virus (Burnet and Anderson, 1946) the most likely interpretation is that in addition to active virus, mumps material derived from chick embryo infections contains much partially disintegrated virus still capable of absorption to the red cell surface but not readily eluted and capable of " bridging " from one cell to another only under the favourable conditions provided by low temperatures. At 37° the cells are stable but have a new surface antigen with which mumps antibody can react and produce agglutination of the cells.

Experiments to test this interpretation are in progress.

SUMMARY.

Human red cells treated with mumps virus are specifically agglutinated by mumps antibody. The reaction can be used as a sensitive quantitative test for antibody in human serum.

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THE THYROTROPHIC HORMONE AND THIOUREA IN REFERENCE TO THE PROBLEM OF "ANTIHORMONES."

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FROM the results of experiments such as those involving the prolonged administration of preparations of the thyrotrophic hormone to rats, Collip (1934) was led to postulate the role of antihormones in normal physiology, a theory subsequently restricted by him to the hormones of the hypophysis. Evidence in support of this concept has been provided by other investigators. Thus Eitel and Loeser (1935), Rowlands and Parkes (1936) and Scowen and Spence (1936)

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have reported the presence of substances in the sera of normal animals which neutralized the effects of anterior pituitary extracts, while Loeser and Trikojus (1937) were able to produce a potent anti-thyrotrophic concentrate from the pooled sera of normal European sheep. Some further support for the theory was given by observations on the modified histological appearance and hormone content of the pituitary glands of animals in receipt of repeated injections of pituitary extracts (e.g. Collip and Anderson, 1934; Thompson and Cushing, 1934; Eitel and Loeser, 1935; Sevringhaus and Thompson, 1939). Furthermore, Anderson and Evans (1938) injected homologous extracts into rats and claimed to have produced an anti-thyrotrophic serum. However, no evidence was obtained by Katzman, Wade and Doisy (1937) for the existence of gonadotrophic or thyrotrophic inhibitory substances in the plasma of rats receiving repeated implantations of anterior pituitary lobes of the same species.

It is now the view of many workers that thyroid refractoriness after prolonged injection of thyrotrophic extracts is in the nature of an immune response to the foreign proteins of the extract, the "anti-hormones" appearing as immune bodies of an unusual type (compare Thompson, 1941). Observations locating the site of formation of the inhibitory principles in the reticulo-endothelial system lend support to this alternative viewpoint (Gordon, Kleinberg and Charipper, 1939; Murphy, Lowther and Pagniello, 1938). Harington and Rowlands (1937) have further shown that the anti-thyrotrophic activity is associated with the pseudo-globulin fraction of the serum proteins. In a crucial experiment Werner (1938) was able to establish that the loss of responsiveness to repeated treatment with the thyrotrophic hormone depends upon the type of extract used. Rowlands and Young (1939) reported a similar finding as regards the antithyrotrophic titre of the serum.

In none of the experiments cited in the literature has an attempt been made to ascertain the effect of homologous hormone on the intact animal after the administration of heterologous extracts to the stage of development of a refractory state. This approach to the problem, as regards the thyrotrophic hormone, has been facilitated for the laboratory animal by the adequately confirmed observations of MacKenzie and MacKenzie (1943) and Astwood and co-workers (1943) that the feeding of certain sulphonamides and thiourylenes causes hypertrophy and hyperplasia of the thyroid glands of rats accompanied by a lowered basal metabolic rate. The explanation at present generally accepted is that the primary action of such compounds is to inhibit the production of thyroid hormone, the concentration of which in circulation is thereby diminished. The pituitary gland, thus relieved from the inhibitory effects of the natural antagonist to its thyrotrophic hormone, is enabled to increase its output of this principle with consequent changes in the cellular structure of the thyroid gland.

The purpose of the experiments recorded below was to ascertain the effects on the guinea-pig thyroid of a thiourylene drug when given concurrently with, and subsequent to, the prolonged administration of heterologous thyrotrophic hormone.

MATERIAL AND METHODS.

Thyrotrophic hormone.

Powdered lyophilized ox anterior pituitary glands were extracted with dilute aqueous sodium bicarbonate, the pH adjusted to 4·5, and after centrifugation

7 volumes of acetone were added. The resulting precipitate was extracted with water, and the hormone recovered from the aqueous solution by the addition of acetone. It was dried over P_2O_5. The preparation was readily and completely soluble in water and assayed approximately 3–5 Junkmann-Schoeller units/mg. (by comparison with a reference specimen donated by Professor Schoeller and containing 34 units/mg.). In previous work it was found that the thyroid glands of guinea-pigs injected daily intraperitoneally with 1 mg. of this preparation had reverted to a normal appearance in almost 100 per cent of the animals after 4–5 weeks.

Thiourea.

This was a thiocyanate free preparation. It was administered orally by pipette as a 9 per cent solution in distilled water twice daily.

Animals.

Immature guinea-pigs from the same stock (Commonwealth Serum Laboratories) and of both sexes and approximately the same age and weight were maintained in an artificially heated room on a diet of bran, fresh greens and milk *ad libitum*, supplemented by 0·2 ml. of vitaminized oil twice weekly. During the latter part of each experiment the sexes in each group were segregated.

Post-mortem examinations.

At the conclusion of each experimental period the animals were killed by a blow on the back of the neck. Thyroids and adrenals were cleanly dissected, weighed after standing 24 hours in formol-saline and prepared for microscopical examination (eosin-haematoxylin stain). In addition, in Exp. 2, the spleens were weighed and median sections fixed and prepared with Leishman stain, while the pituitary glands, assembled for each group, were immediately treated with acetone and then washed with peroxide-free ether, dried over P_2O_5 and retained for assays of the thyrotrophic hormone.* The mean acinar cell heights of the thyroid glands were estimated by a technique essentially similar to that described by Griesbach and Purves (1943), using an etched scale on thin glass and a magnification of 1000. The values given in the tables represent the mean values of 50 acini per section cut longitudinally near the centre of each gland.

Statistical treatment of results.

The data have been analysed throughout, using the analysis of variance technique, and the standard errors (S.E.) of the means evaluated from the error or within-group variance. In the tables showing significant differences between means, the values of t derived are associated with the same number of degrees of freedom as the error variance in the appropriate experiment: in Exp. 1, $\nu = 30$; Exp. 2, $\nu = 65$; Exp. 3, $\nu = 18$. The criterion of significance is that P, the probability of a purely chance occurrence of a difference between any two means, should be less than 0·05.

* The method appropriate to the assay of these small quantities is that using the day-old chick. We are at present unable to procure regular supplies of standard test material, and therefore do not report the results in this communication.

EXPERIMENTAL PROCEDURES AND RESULTS.

In a preliminary experiment, which is not reported in detail, 41 guinea-pigs in 7 groups were given thiourea with and without thyrotrophic hormone for 7 weeks, or for 2 weeks following pretreatment with thyrotrophic hormone for 5 weeks. The daily constant dosages of thiourea and thyrotrophic hormone were 160 mg. (80 mg. each morning and afternoon) and 1 mg. (intraperitoneally) respectively. Under these treatments the animals in all groups gained satisfactorily in weight and showed no toxic symptoms throughout the experiment, which was designed to ascertain suitable dosages and periods of administration. The main impression gained from this experiment was that pretreatment with thyrotrophic hormone caused a lower level of response to thiourea as judged from the microscopic examination of the thyroid glands. The three most relevant groups with larger numbers of animals were accordingly reinvestigated in the following experiment.

Experiment I : Thyrotrophic Hormone Followed by Thiourea for Two Weeks.

Group I.—Thyrotrophic hormone plus thiourea (11 animals). The hormone was given as for Group II but for $7\frac{1}{2}$ weeks, supplemented by thiourea as for Group III during the final two weeks of this period.

Group II.—Thyrotrophic hormone (10 animals). 1 mg. of the preparation was administered daily, intraperitoneally, for $5\frac{1}{2}$ weeks on 6 days per week.

Group III.—Thiourea (12 animals). This was given twice daily at a constant level of 80 mg. for two weeks (24 administrations).

The animals in the three groups were placed under experimental conditions at the same time, and the respective treatments so arranged that the animals could be despatched as groups on successive days at the conclusion of the experiment. Body-weights were recorded thrice weekly, and the mean values for each group are set out in Fig. 1. In Table I the remaining data are given as mean values together with their standard errors and significant differences.

Results.

The mean body-weights increased in all groups throughout the experiment (Fig. 1), although there was a reduction in the rate of increase during the period of administration of thiourea (Groups I and III). As can be seen from Table I, there is no significant difference between the mean acinar cell heights of the thyroids of Groups I and II, but the corresponding value for Group III is significantly higher than that of both these groups. The general microscopic appearances of the thyroid sections were in conformity with this finding, those of Group III being characterized by hyperplasia and colloid absorption, whereas with the exception of one mildly active gland in each group, those of Groups I and II were of normal type. These results would indicate that the states of the thyroid and pituitary glands, after administration of thyrotrophic hormone to a refractory phase, are such that no apparent response is produced in the thyroid gland by otherwise effective amounts of thiourea. The matter is further discussed at a later stage.

The mean thyroid weights (mg./100 g.) of the group receiving thiourea following hormone treatment and that receiving hormone alone (Groups I and II) were greater than that of the thiourea-treated group (III), and the difference is significant for Groups I and III. It is possible that the thyroids of some of the animals

in Group I, although histologically inactive, had not quite returned to normal size before the beginning of treatment with thiourea (compare Trikojus and Ellis, 1939), while owing to the lag in response (compare Larson, Keating, Peacock and Rawson, 1945) the administration of this drug for the short period of two weeks should not have induced appreciable thyroid enlargement in Groups I and III. A normal control group was not placed concurrently with the animals in this experiment, carried out in the summer months, although the control

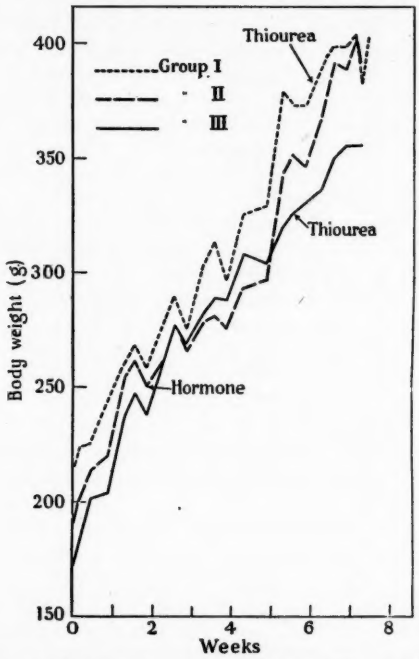

FIG. 1.—Body-weight curves of guinea-pigs receiving thiourea (80 mg. twice daily) for 2 weeks (Group III), thiourea during final 2 weeks of bovine thyrotrophic hormone treatment (1 mg./day) for 7½ weeks (Group I) and hormone for 5½ weeks (Group II). Thiourea treatment in both Groups I and III commenced at 5½ weeks. Mean data from Exp. 1.

group of 10 animals of similar average weight used in Exp. 3 and assembled during the same period of the year had a mean thyroid weight of 9·3 mg./100 g. (S.E., 0·51) and mean acinar cell height of 3·79μ (S.E., 0·24). A possible seasonal influence may be noted in the significantly greater mean thyroid weight and acinar cell height for the control group in Exp. 2, conducted during winter, although in a room maintained at approximately constant temperature (14·72 mg./100 g. (S.E., 0·88); 4·81μ (S.E., 0·23)). The difference of the mean thyroid weights in the two control groups is 5·42 (S.E., 1·57) with $t = 3{\cdot}45$ and $P < {\cdot}01$, and that of the acinar cell heights 1·22 (S.E., 0·33) with $t = 3{\cdot}69$ and $P < {\cdot}001$.

It is noteworthy that the adrenal glands, although of normal appearance, were significantly reduced in size even after this short period of treatment with

TABLE I.—*The Effects of Prolonged Treatment with Bovine Thyrotrophic Hormone (T.H.), Followed by Administration of Thiourea for Two Weeks on the Thyroid and Adrenal Glands of the Guinea-Pig. Mean Data from Experiment 1 and Their Statistical Treatment. Thiourea, 160 mg., Hormone, 1 mg., Daily.*

Group No.	Treatment.	Number of animals.	Mean final body-weight. g.	Thyroid acinar cell heights. μ.		Thyroids. mg./100 g.		Adrenals. mg./100 g.	
				Mean.	S.E.	Mean.	S.E.	Mean.	S.E.
I	T.H., 7¼ weeks; thiourea, final 2 weeks only	11	402	4·14	0·22	15·33	0·87	50·28	2·30
II	T.H., 5¼ weeks	10	390	3·70	0·23	14·47	0·91	57·12	2·42
III	Thiourea, 2 weeks	12	355	5·73	0·21	11·41	0·83	42·40	2·21

Significant Differences:

Comparisons of groups.	Acinar cell height. μ.				Thyroids. mg./100 g.				Adrenals. mg./100 g.			
	Diff.	S.E.	t.	P.	Diff.	S.E.	t.	P.	Diff.	S.E.	t.	P.
I–II	− 1·60	0·31	5·24	< ·001	− 3·92	1·20	3·26	< ·01	− 6·84	3·34	2·05	·05
I–III	− 2·03	0·31	6·50	< ·001	+ 3·06	1·23	2·48	·02	+ 7·88	3·19	2·47	·02
II–III									+ 14·72	3·27	4·50	< ·001

thiourea. (See Table I; Groups I and III compared with Group II.) The significantly higher value for the mean adrenal weight in Group I compared with Group III may be explained as for the difference in thyroid weights between these two groups. The changes in the adrenal glands, both microscopic and macroscopic, became much more apparent after the longer periods of administration of thiourea in Exp. 2.

Experiment 2: Involving Longer Treatment with Thiourea than in Exp. 1.

In view of the observations of MacKenzie and MacKenzie (1943) that older rats were less sensitive to sulphaguanidine than young animals, and of the fact that goitrous changes in the thyroid of the chick do not appear until 5 days' treatment with thiouracil (Larson *et al.*, 1945), it was decided in this, the main experiment, to extend the treatment with thiourea, after pretreatment with thyrotrophic hormone, to 4 weeks. Furthermore, the twice daily dose of thiourea was graded on the basis of 30 mg./100 g. body-weight (with adjustment at each 50 g. level). The thyrotrophic hormone was administered by the intraperitoneal route, 1 mg. per day, as in the previous experiment. The animals (74) were divided into 8 groups, one of which (Group VI) was intended to provide for possible losses during the prolonged administration of thiourea to Group I, but since only one animal succumbed, the treatment for the smaller group was continued for a further 5 weeks.

Group I.—Thyrotrophic hormone and thiourea for 10 weeks (10 animals—one died during experiment).

Group II.—Thiourea for 10 weeks (10 animals).

Group III.—Thyrotrophic hormone for 10 weeks (10 animals).

Group IV.—Thyrotrophic hormone for 6 weeks (10 animals).

Group V.—Thyrotrophic hormone for 10 weeks with thiourea additionally during last 4 weeks (10 animals).

Group VI.—Thyrotrophic hormone and thiourea for 15 weeks (4 animals).

Group VII.—Thiourea for 4 weeks (10 animals).

Group VIII.—Normal controls (10 animals).

With the exceptions of those in Groups VII and VIII, all animals were placed under experimental conditions at the same time and treatment commenced after an initial observation period of one week. The animals in Group VII were selected from the same stock after 5 weeks, so that their weights approximately covered the weight variation in the treated groups. One-half of the animals in the normal control group was also selected at this time, and the remainder 8 weeks after the beginning of the experiment. Body-weights were recorded daily for animals receiving thiourea and thrice weekly in the other groups. Treatment was given on 6 days per week, and was arranged to allow for the despatch of all groups excepting IV and VI on three successive days. The body-weights are represented in graphical form in Fig. 2, while the other main data together with the results of their statistical treatment are recorded in Tables II and IIA.

Results.

(a) *Body-weights.*—After a preliminary period, animals receiving thiourea as a supplement did not gain in weight as rapidly as those in the other groups. This is apparent from Fig. 2, both for the long term groups, particularly during the

latter stages, and for the short-term group where a comparison can be made with the normal control group (curves inset on Fig. 2).

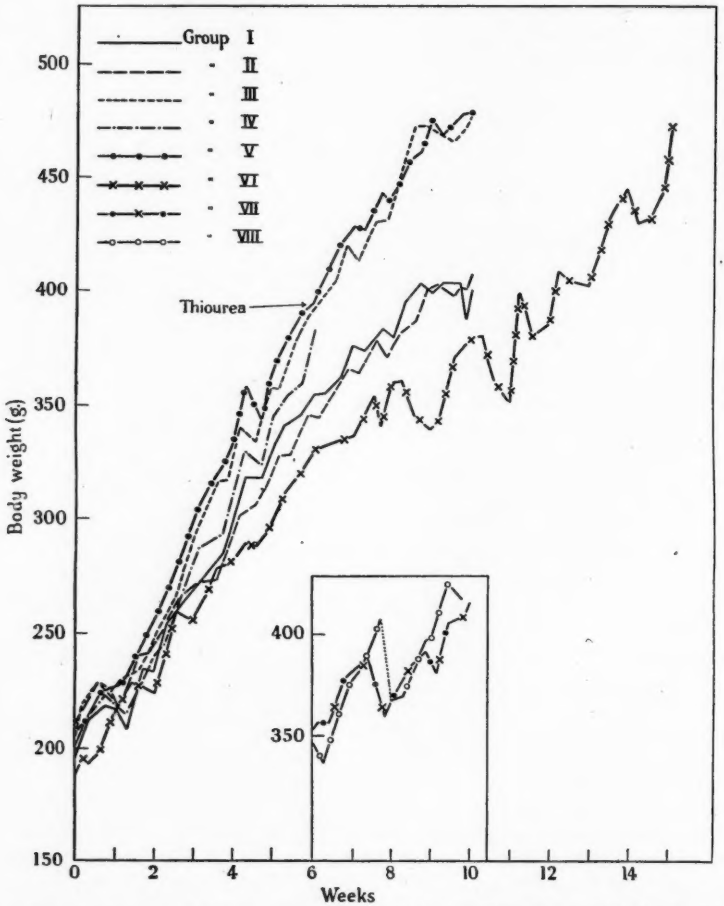

FIG. 2.—Body-weight curves of guinea-pigs receiving thiourea (T.U., 30 mg./100 g. body-weight twice daily) or bovine thyrotrophic hormone (T.H., 1 mg./day) or both together for 4 to 15 weeks. Group I (T.U. and T.H. for 10 weeks); Group II (T.U. for 10 weeks); Group III (T.H. for 10 weeks); Group IV (T.H. for 6 weeks); Group V (T.H. for 10 weeks; T.U. for final 4 weeks); Group VI (T.U. and T.H. for 15 weeks); Group VII (T.U. for 4 weeks); Group VIII (normal controls). Curves for last two groups inset (note break in curve for Group VIII where group increased in size). Mean data from Exp. 2.

(b) *Macroscopic and microscopic findings.*—The animals in all groups were in good condition at the conclusion of the experiment. No gross differences were apparent at autopsy between the normal control group (VIII) and those receiving

TABLE II.—*The Effects of Prolonged Administration of Thiourea and Bovine Thyrotrophic Hormone (T.H.), and Thiourea Following Pretreatment with the Hormone, on the Thyroid Gland and Organ Weights of the Guinea-pig. Mean Data from Experiment 2. Thiourea, 60 mg./100g. Body-weight, and Hormone, 1 mg., Daily.*

Group No.	Treatment.	Number of animals.	Mean final body weight. g.	Thyroid acinar cell heights. μ		Thyroids. mg./100 g.		Adrenals. mg./100 g.		Spleen. mg./100 g.	
				Mean.	S.E.	Mean.	S.E.	Mean.	S.E.	Mean.	S.E.
I	T.H. + thiourea, 10 weeks	9	400	7·24	0·24	35·54	1·57	35·71	2·73	175·66	14·63
VI	T.H. + thiourea, 15 weeks	4	465	6·10	0·36	29·28	2·35	26·70	4·09	165·50	21·04
II	Thiourea, 10 weeks	10	408	7·25	0·23	32·27	1·49	28·58	2·59	183·61	13·[illegible]
III	T.H., 10 weeks	10	476	4·33	0·23	15·32	1·49	58·53	2·59	149·14	13·85
IV	T.H., 6 weeks	10	381	4·34	0·23	15·07	1·49	53·93	2·59	139·44	13·88
V	T.H., 10 weeks ; thiourea, final 4 weeks only	10	478	7·00	0·23	19·30	1·49	32·29	2·59	176·85	13·88
VII	Thiourea, 4 weeks	10	411	8·40	0·23	29·11	1·49	38·95	2·59	201·62	13·88
VIII	Normal controls	10	417	4·81	0·23	14·72	1·49	55·67	2·59	151·85	13·88

TABLE IIA.—*Statistical Treatment of the Data from Experiment 2 (see Table II).*

	Acinar cell heights. μ.				Thyroids. mg./100 g.				Adrenals. mg./100 g.			
	Diff.	S.E.	t.	P.	Diff.	S.E.	t.	P.	Diff.	S.E.	t.	P.
Hormone alone.												
III–VIII	−	..	..	..	−	..	..	..	−	..	..	..
IV–VIII	−	..	..	..	−	..	..	..	−	..	..	..
Thiourea alone	+				+				−			
II–VIII	2·44	0·32	7·52	< ·001	17·55	2·10	8·35	< ·001	27·09	3·66	7·41	< ·001
VII–VIII	+ 3·59	0·32	11·07	< ·001	+ 14·39	2·10	6·85	< ·001	− 16·72	3·66	4·57	< ·001
II–VII	− 1·15	0·32	3·55	< ·001	−	..	..	..	− 10·37	3·66	2·84	< ·01
Hormone + thiourea	+				+				−			
I–VIII	2·43	0·33	7·31	< ·001	20·82	2·16	9·65	< ·001	19·96	3·76	5·31	< ·001
VI–VIII	+ 1·29	0·43	3·01	< ·01	+ 14·56	2·78	5·24	< ·001	− 28·97	4·84	5·99	< ·001
I–VI	+ 1·14	0·44	2·63	< ·01	+ 6·27	2·82	2·22	< ·05	−	..	..	..
I–II	−	..	..	..	−	...	..	..	−	..	..	..
I–VII	− 1·16	0·33	3·47	< ·001	+ 6·43	2·16	2·98	< ·01	−	..	..	..
Pretreatment with hormone.	+				+				−			
V–VIII	2·19	0·32	6·75	< ·001	4·58	2·10	2·18	< ·05	23·38	3·66	6·39	< ·001
V–VII	− 1·40	0·32	4·32	< ·001	− 9·81	2·10	4·67	< ·001	−	..	..	..

thyrotrophic hormone alone (Groups III and IV). In contrast to these three groups, those receiving thiourea were characterized by greatly enlarged and frequently hypervascular thyroids with, in many cases, a pronounced isthmus; the spleens appeared a much darker colour and slightly engorged, while the adrenals were obviously smaller, particularly after prolonged treatment, and varied from grey to brown or greenish brown in colour.

Microscopically the thyroid glands of Groups III and IV could not be distinguished in general appearance from those in the normal control group, whereas those from animals which had received thiourea were uniformly hyperplastic, with varying degrees of colloid absorption and papillary infolding. Considered as groups the thyroids from Group VII (thiourea for 4 weeks) showed the most advanced modifications of structure, even more than those from Group I (thiourea and thyrotrophic hormone for 10 weeks) and Group II (thiourea for 10 weeks), while less profound changes were exhibited by the thyroids in Groups V and VI. A more quantitative interpretation of the thyroid changes appears under (c) below.

When compared with those of the controls and of the animals receiving hormone alone, the sections of the adrenals of all groups receiving thiourea displayed a less marked contrast between medulla and cortex. The general reduction in size of the adrenals in these animals could be associated with a diminution in lipoid content, in total depth of the cortex and in the size of the cortical cells.

The sections of the spleens of those animals which had received thiourea, alone or in conjunction with hormone, were characterized by prominent sinuses, greatly congested pulp, and lack of prominence of the cords. There was very little lymphoid hyperplasia; the follicles were widely separated and clearly defined. On the other hand, the spleens of the animals receiving hormone alone showed prominent cords, no congestion of the pulp, less definition of the sinuses and pronounced lymphoid hyperplasia. The spleens of the animals in the control group (Group VIII) were all of normal histological appearance.

(c) *Quantitative data and their statistical treatment.*—See Tables II and IIA.

Thyroid acinar cell heights.—The values for the groups receiving thyrotrophic hormone alone for either 6 or 10 weeks are not significantly different from that of the control group (VIII). On the other hand, the height was increased in all the groups receiving thiourea either alone or in conjunction with thyrotrophic hormone. The increase was not so marked in the case of animals treated with thiourea for 10 weeks as for those treated for 4 weeks (Groups II and VII), while for those given the hormone and thiourea together for 10 and 15 weeks a correspondingly less marked increase after prolonging the treatment is apparent. There is no difference between the values of the two groups (I and II) in which thiourea was given alone or in conjunction with thyrotrophic hormone for 10 weeks; however, when the hormone was given for 6 weeks prior to the administration of thiourea for 4 weeks, the effect of this pretreatment did not abolish the response to thiourea, as was the case in Exp. 1, but the increase in cell height was significantly reduced.

Thyroid weights.—While prolonged treatment with thyrotrophic hormone alone did not affect the thyroid weight, the administration of thiourea led to a marked increase in all the groups receiving this drug. There is no difference in the values of the groups treated with thiourea for 4 or 10 weeks, but there was less increase after 15 weeks than after 10 weeks when hormone and thiourea were

given together. Thiourea for 4 weeks following hormone treatment (Group V) also brought about considerable thyroid enlargement, but this was less than when thiourea alone was administered for the 4 weeks.

Adrenal weights.—The administration of hormone alone effected no change in the adrenal weight, but thiourea alone or in association with hormone caused a progressively greater decrease in weight with length of treatment, although there was no difference between the groups receiving both together for 10 and 15 weeks. Pretreatment with hormone did not affect the reduction in weight brought about by thiourea during 4 weeks.

Spleens.—The spleens showed such wide individual variation that no difference between sample means is significant, but the spleens were slightly enlarged in those animals receiving thiourea. If all the values are grouped into two samples, those receiving thiourea and those not, the mean weights of the spleens are respectively $182{\cdot}88 \pm 7{\cdot}27$ mg./100 g. and $146{\cdot}81 \pm 6{\cdot}92$ mg./100 g., and the difference is $36{\cdot}07 \pm 10{\cdot}04$, giving $t = 3{\cdot}45$ and $P \lessapprox {\cdot}001$.

Experiment 3: Effect on Guinea-pig Thyroid of Short-term Injections of Bovine Thyrotrophic Hormone.

In order to derive an estimation of the level of production of thyrotrophic hormone by the guinea-pig, under the influence of the doses of thiourea used in this investigation, a group of 10 animals was injected daily with 1 mg. of the preparation of bovine thyrotrophic hormone for 7 days. The thyroids were removed on the 8th day and submitted to microscopic examination, and compared with those of a group of 10 normal controls and the group in Exp. 1 receiving thiourea for two weeks (Group III). At the beginning of the test the body-weights of the treated group and the control averaged 366 g. and 363 g., being thus comparable with those of the groups in Exps. 1 and 2 at the stage of administration of thiourea for two and four weeks respectively.

Results.

The results are set out in Table III. There is no difference in the mean thyroid weights of the two groups, which can probably be correlated with the lowered response of the thyroid of the more mature guinea-pig to the pituitary hormone, but the mean acinar cell height was increased by the hormone to a level

TABLE III.—*The Effects of 7 Daily Injections of 1 mg. Bovine Thyrotrophic Hormone (T.H.) on the Thyroid Gland of the Guinea-pig. Mean Data from Exp. 3 and Their Statistical Treatment.* (*See also Table I (Group III)*).

Group No.	Treatment.	Number of animals.	Mean final body-weight. g.	Thyroid acinar cell heights. μ.		Thyroids, mg./100 g.	
				Mean.	S.E.	Mean.	S.E.
I	T.H.—7 days .	10	419	5·96	0·24	9·91	0·51
II	Normal controls	10	413	3·79	0·24	9·30	0·51

Comparison of groups.	Diff.	S.E.	t.	P.	Diff.	S.E.	t.	P.
I–II	+ 2·17	0·34	6·43	< ·001	—	..	..	..

comparable with that produced by two weeks' treatment with thiourea. Assuming a lag period in the response to thiourea, the daily output of excess endogenous hormone resulting from the effects of the doses of the drug used in this investigation was thus qualitatively equivalent to the daily injected dose of the bovine thyrotrophic hormone.

DISCUSSION.

Under the two sets of experimental conditions recorded in this paper it is clear that preliminary treatment of the guinea-pig with a heterologous preparation of the thyrotrophic hormone to the stage of development of a refractory thyroid state prevents, or significantly reduces, depending upon the period of administration, the normal pituitary-thyroid response to a thiourylene drug, thiourea, as judged by acinar cell height measurements of the thyroid glands. A reduction in the degree of hyperplasia is also apparent from the data on the mean thyroid weights in Exp. 2 (Groups V and VII). In explanation it may be suggested that some protection from the effects of endogenous hormone has been afforded the animal by immune bodies produced as a result of continued adminstration of the heterologous extract. Although the guinea-pig is not suitable for the production of high-titre antisera, Bruner and Starr (1936) and Werner (1938) found the refractory thyroid in this animal insensitive to 400 times and 20 times, respectively, the initiating dose of the extract used. If the rate of production of thyrotrophic hormone in the normal guinea-pig treated with thiourea as in these investigations is of the order indicated by the results of Exp. 3, it may be concluded that only slight protection against the endogenous hormone has been provided.

There was no difference between the mean acinar cell heights and thyroid weights of the two groups of animals in Exp. 2 receiving thiourea alone or thiourea plus bovine thyrotrophic hormone for 10 weeks. In both of these groups there would have been a low level of synthesis of thyroid hormone from early in the period of administration, a possible complicating factor being thereby eliminated. It would therefore seem that the protective effect of the "antihormone" can be overcome, and that it leads merely to an extension of the lag period before the full response to thiourea occurs.

Although the mean thyroid data for the two groups of animals receiving hormone alone for 6 and 10 weeks in Exp. 2 were within normal limits, it would be consistent with the above argument, and with the observations of Kupperman, Meyer and Finerty (1942) and Kupperman and Meyer (1945) on the effects of antigonadotrophic serum, to assume a slightly higher than normal level of production of endogenous pituitary hormone by these two groups to allow for its partial neutralization in the blood-stream. The results of the assays of the pituitary glands collected in Exp. 2 should throw further light on the mechanisms involved, although it is true that there would still be lacking evidence of the functional state of the thyroid gland other than that provided by the thyroid weights and mean acinar cell heights.

There is some evidence from our results of a decline in the thyroid response to thiourea with time, this being independent of the heterologous hormone, and in accord with the experiences of Kennedy and Purves (1941) with brassica seeds and Bielschowsky (1944) with allylthiourea (see also Astwood *et al.*, 1943). On the other hand, the reduction in relative size of the adrenal glands became more

pronounced as the treatment with thiourea was prolonged. A specific adrenal mechanism may thus be involved apart from that dependent upon the thyroid-adrenocortical interrelationship, as suggested by Baumann and Marine (1945*a*) in explanation of the comparable effects obtained by them with thiouracil in the adrenal gland of the rat. Other investigators (Richter and Clisby, 1942; Williams, Bissell, Jandorf and Peters, 1944) using the rat and thiourylene drugs could observe no changes in the adrenal glands of their animals. Kennedy and Purves (1941), however, noted adrenal hypertrophy and an increase in the cortical lipoids associated with the goitrous changes produced by a brassica seed diet. In the latter experiments the presence of substances affecting the adrenal gland, apart from the goitrogenic agent, cannot be excluded. There is a contrast between our observations on the splenic changes induced by thiourea and those of Baumann and Marine (1945*b*), who reported a marked shrinkage in this organ after the continued feeding of thiouracil to the rat.

SUMMARY.

The effects of thiourea in the presence and absence of a heterologous (bovine) preparation of the thyrotrophic hormone have been studied in two experiments involving 107 guinea-pigs.

Pretreatment with hormone to a thyroid refractory stage has been shown to abolish or significantly reduce the effects on the thyroid gland of thiourea subsequently administered for 2 and 4 weeks respectively. It is suggested that this inhibitory action is only slight and temporary in nature, and that it is related to the development of immune bodies to the foreign extract, the effects of which could be overcome by further extension of the period of treatment with thiourea.

Changes in the other organs were most pronounced in the adrenal glands, which exhibited cortical atrophy and dark pigmentation, together with a progressive decrease in relative size as the administration of thiourea was prolonged. The spleens of the thiourea treated animals were darker and had a slightly greater relative weight than those not receiving this drug. Microscopic differences were also apparent.

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AN ELECTRON MICROSCOPE STUDY OF THE CONTENTS OF HAIR CELLS FROM LEAVES DISEASED WITH TOBACCO MOSAIC VIRUS.

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SINCE the original description of purified tobacco mosaic virus (Stanley, 1935), a considerable body of experimental evidence has accumulated which indicates that the minimal infective units of this virus are rod-like particles about 15 by 280 mμ. in size (Bernal and Fankuchen, 1941; Eriksson-Quensel and Svedberg, 1936; Lauffer, 1940, 1943*a*, 1943*b*, 1944*a*, 1944*b*; Lauffer and Stanley, 1938, 1943; Loring and Stanley, 1937; Loring, Lauffer and Stanley, 1938; Stanley, 1936, 1937*a*, 1937*b*, 1937*c*, 1940; Stanley and Anderson, 1941; Wyckoff, Biscoe and Stanley, 1937). Much of this work has had to do with the biological, chemical and physical interrelationships of purified preparations of tobacco mosaic virus consisting essentially of these rod-like particles. Recently Lauffer (1943*b*) demonstrated that the sedimentation constant of the infectious principle of tobacco mosaic is the same, within a probable error of 6 per cent, as that of the rod 15 by 280 mμ. in size. Lauffer's results prove that, in the case of the present material, the sole carriers of virus activity cannot possibly be particles any smaller than half the size of these rods. Despite the wealth of experimental evidence relating tobacco mosaic virus activity directly to the rods 15 by 280 mμ. in size and the complete absence of experimental data correlating virus activity with a smaller particle, Bawden and Pirie (1945) and

Bawden (1946) continue to state that it is highly probable that the rods 15 by 280 mμ. are aggregates, and that the primary virus particle is much smaller and not greatly elongated. It must be recognized that, as with any biologically active material, the possibility always exists that at some future time, perhaps through new methods of treatment of virus preparations or through new inoculation techniques, virus activity may be found associated with smaller particles. However, it seems unnecessary to make definitive postulations in this direction without supporting experimental data, and at this time the necessary supporting experimental data are lacking in the case of purified preparations of tobacco mosaic virus. There is, of course, complete accord that the rods 15 by 280 mμ. in size can be caused to aggregate end-to-end, and that this aggregation is, to a certain extent, irreversible. Bawden and Pirie (1945) as well as Sigurgeirsson and Stanley (1946) have obtained preparations of small particles from concentrates containing tobacco mosaic virus, and in both instances these were found to be essentially devoid of virus activity. Since these preparations of small particles possess little or no virus activity, it seems unwise to refer to them as " virus " as Bawden and Pirie have done, for this term should be restricted to the material which has been demonstrated to possess a characteristic and maximal virus activity.

Bawden (1946) appears to believe that tobacco mosaic virus exists within plant cells in the form of small, essentially spherical particles, and that " aggregation to form elongated particles may be an inescapable result of purification," yet in the same article he presents evidence for the existence within plant cells of crystalline inclusions which can be converted directly to needle-like crystals containing the rod-like particles. Although but little attention has been given to the nature of the virus in the plant cells and in untreated infectious juice, some evidence that rod-like particles actually exist within the plant cells and in untreated infectious juice is provided by the work of Beale (1937) on the nature of the virus within living cells, and the demonstration by Wyckoff, Biscoe and Stanley (1937) that untreated infectious juice contains a component which sediments in the ultracentrifuge at the same rate as purified preparations of virus consisting of the rods 15 by 280 mμ. Sigurgeirsson and Stanley (1946) have also demonstrated, by means of the electron microscope, that most of the rod-like particles present in untreated infectious juice are 15 by 280 mμ. in size. In order to secure further information concerning the virus present within infected cells a study was made, by means of the electron microscope, of the contents of hair cells of leaves of Turkish tobacco plants infected with tobacco mosaic virus. Ordinary electron micrographs do not give good pictures because of the poor electron scattering contrast between the virus particles and the background of plant materials. Therefore, in the present investigation, the metal shadow-casting technique of Williams and Wyckoff (1944) was employed because it makes it possible to distinguish readily the virus particles from the plant materials.

METHODS.

Hair cells were cut off with a razor blade from the leaves of Turkish tobacco plants which had been infected for 25 days with tobacco mosaic virus. About a dozen cells were placed on a glass slide and gently broken with a glass rod. The extruded contents were immediately transferred to electron micrograph

FIG. 1.—Electron micrograph of the contents of hair cells from leaves of Turkish tobacco plants diseased with tobacco mosaic virus. The mount was prepared with gold by the shadow-casting technique. The magnification is 32,500.

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screens with a drop of distilled water. The screens had previously been mounted on glass slides and covered with a thin film of collodion. The preparations were allowed to dry, and the slides were then dipped into distilled water to remove soluble salts.

The glass slides were placed in a high vacuum system (less than 10^{-6} mm. pressure), and a known amount of gold was deposited obliquely on them from a hot tungsten filament source. The slides were at an angle of 15° and were 20 centimetres from the source of gold. It is estimated that the average thickness of gold over the surface of the sample was 8Å.

The samples were observed in an RCA Console Model (type EMC–1) electron microscope having a magnification of 5800 times. Representative pictures were enlarged photographically, and the length of every particle in the pictures having a diameter of about 15 mμ. was measured.

RESULTS.

A typical electron micrograph of the contents of hair cells from leaves of Turkish tobacco plants diseased with tobacco mosaic virus is shown in Fig. 1. The size distribution of the particles present in Fig. 1 and in similar micrographs, represented in percentages of the total number of particles counted (800), is illustrated in Fig. 2. It is apparent from Fig. 1 and 2 that most of the particles

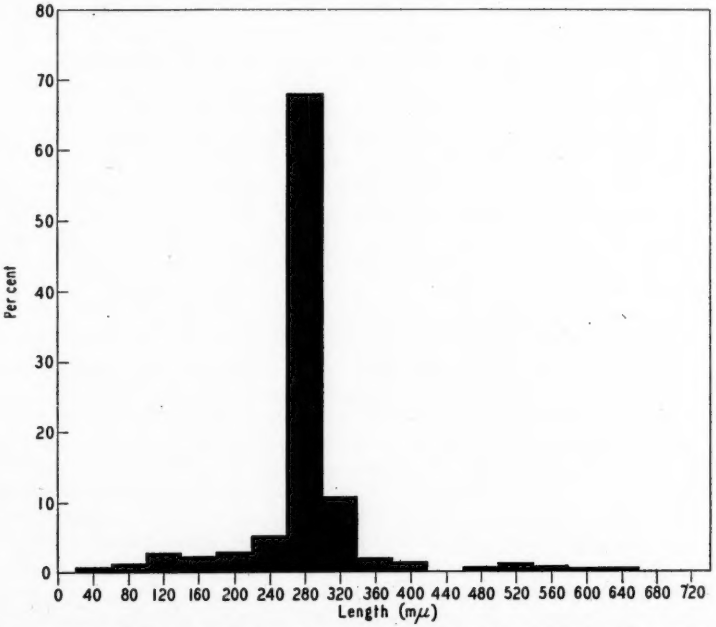

FIG. 2.—Size distribution of rod-shaped particles present in the contents of hair cells from leaves of Turkish tobacco plants diseased with tobacco mosaic virus. The distribution is represented in percentages of the total number of particles counted, which was 800. Prepared from Fig. 1 and similar micrographs.

(number per cent 68, weight per cent 67) are approximately 280 mμ. in length. It seems unlikely that the particles having a length of 280 mμ. represent fortuitous aggregations of smaller particles. It can be shown, on the basis of probability considerations and from the diffusion constants of hypothetical smaller particles, that no appreciable aggregation of such particles to form rods 280 mμ. in length could take place from the time the hair cells were broken until the final drying of the sample. Furthermore, it can be shown that if such polymerization should eventually take place, the size distribution of the particles so formed would be much broader than that illustrated in Fig. 2. Since the method used to secure the contents of the hair cells probably involved only mild agitation at the molecular level, it seems reasonable to conclude that the virus particles exist in cells with approximately the size distribution represented in Fig. 2. This size distribution is essentially the same as that found by Sigurgeirsson and Stanley (1946) for rod-shaped particles in the untreated juice pressed from mosaic-diseased Turkish tobacco plants.

Electron micrographs of the contents of hair cells from leaves of normal Turkish tobacco plants have been made, and these do not show the presence of the rod-shaped particles which are characteristic of mosaic diseased plants.

DISCUSSION.

The results of the present investigation and those of Sigurgeirsson and Stanley (1946) demonstrate that the contents of hair cells or the juices pressed from leaves of Turkish tobacco plants diseased with tobacco mosaic virus contain rod-like particles and that most of these particles are about 280 mμ. in length. Since virus activity has been found to be a specific property of this material, and has not been demonstrated to be associated with particles of smaller size, it seems reasonable to conclude that these particles, 15 by 280 mμ. in size, represent tobacco mosaic virus. There appears to be little or no justification for the assumption, which has been supported by Bawden and Pirie (1945), that tobacco mosaic virus exists within plant cells in the form of small, essentially spherical particles, which aggregate during purification of the virus to yield the rods 15 by 280 mμ. in size. The existence of rods of shorter length in the contents of infected hair cells or in untreated infectious juice can be explained by the break-up of rods having lengths of 280 mμ. or multiples thereof. Preparations of such short rods were obtained by Bawden and Pirie (1945) and by Sigurgeirsson and Stanley (1946), and were found to possess little or no virus activity.

It should be emphasized that the size and shape of tobacco mosaic virus are known with considerable precision, despite the assertion by Bawden (1946) "that the size of no virus particle is known with certainty," and that the size of tobacco mosaic virus "remains a matter of speculation and controversy." Bawden's further statement that the relative sizes of viruses as shown in charts, such as the ones published from this laboratory (Stanley, 1938*a*, 1938*b*, 1939; Stanley, Knight and De Merre, 1945), "are largely fallacious, because they suggest that our knowledge of this subject is much more definite than it actually is" seems unwarranted. The values recorded in the original chart, and in subsequent revisions, were the best that were available in the literature at the time and were, in every case, the direct result of experiments by competent investigators in laboratories throughout the world. The charts have served to

provide an indication of the relative sizes of different viruses as determined by experiments, and have been found very useful by many workers in the virus field. Bawden's statements relative to the sizes of viruses are rather inconsistent, for in one paper he refers to the wide limits of the sizes attributed to tobacco mosaic virus (Bawden, 1946), whereas in another publication (Bawden and Pirie, 1945) the statement is made that "since 1941 Stanley and Lauffer have postulated a basic particle or molecule about 15 mμ. thick and 280 mμ. long." It should be noted that this size for the minimal infective unit of tobacco mosaic virus remains unchanged and that it can hardly be regarded as a postulation, for results such as those recorded in the present paper, as well as many other experimental results, provide considerable experimental evidence that tobacco mosaic virus is approximately 15 by 280 mμ. in size, and that it exists as such within the cells of diseased plants.

SUMMARY.

Electron micrographs, prepared by the gold shadow-casting technique, of the contents of hair cells from leaves of Turkish tobacco plants diseased with tobacco mosaic virus show rod-like particles, 68 per cent of which are about 280 mμ. in length. Similar micrographs prepared from hair cells of leaves of normal Turkish tobacco plants do not show these characteristic rod-like particles. It is concluded that the rods, 15 by 280 mμ. in size, which have been found to represent the minimal infective units of tobacco mosaic virus, occur within the cells of plants infected with tobacco mosaic virus. The data do not support the suggestion of Bawden and Pirie that the primary virus particle is small and not greatly elongated.

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